Relevance of MUC1 splice variants in pancreatic carcinogenesis

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List of Abbreviations

A

aa	amino acid
APC	Adenomatous polyposis coli
ABCC1	ATP-Binding Cassette Sub-Family C Member 1

B

BMI	Body mass index
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2

C

CA19-9	Carbohydrate antigen 19-9
CD133	Cluster of differentiation 133
CD24	Cluster of differentiation 24
CD44	Cluster of differentiation 44
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CHC	Chlorinated hydrocarbon
CSCs	Cancer stem cells
CT	Computed tomography
CTGF	Connective tissue growth factor

D

DNA	Deoxyribonucleic acid

E

EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ESA	Epithelial-specific antigen

F

FAMMM	Familial atypical multiple mole melanoma

FAP	Familial adenomatous polyposis

G

GSK-3β	Glycogen synthase kinase 3β

H

HSP70	Heat shock proteins 70
HSP90	Heat shock proteins 90

I

IARC	International Agency for Research on Cancer
IL-1	Interleukin 1
IL-4	Interleukin 4
IPMNs	Intraductal papillary mucinous neoplasms

K

KRAS	Kirsten rat sarcoma viral oncogene homolog

M

MAPK	Mitogen-activated protein kinase
MCN	Mucinous cystic neoplasm
miRNA	microRNA
MRI	Magnetic resonance imaging
mRNA	messenger RNA
MRP1	Multidrug resistance protein 1
MUC	Mucin
MUC1-CD	MUC1 cytoplasmic domain

N

NCI	National Cancer Institute

O

OR	Odds ratio

P

PAH	Polycyclic aromatic hydrocarbons
PALB2	Partner and localizer of BRCA2
PanINs	Pancreatic intraepithelial neoplasms
PanNETs	Pancreatic neuroendocrine tumors
PC	Pancreatic cancer
PCN	Pancreatic cystic neoplasms
PDAC	Pancreatic ductal adenocarcinomas
PDGF-B	*Platelet-derived growth factor subunit B*
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3´-kinase
PKCδ	Protein kinase C δ
PP	Pancreatic polypeptide
PTS-rich tandem repeat	Proline, threonine and serine rich tandem repeat

R

RAF	Rapidly Accelerated Fibrosarcoma
RalGDS	Ral guanine nucleotide dissociation stimulator
Rb-1	Retinoblastoma 1
RNA	Ribonucleic acid

S

SCNs	Serous cystic neoplasms
SEA	Sea urchin sperm protein, Enterokinase and Argin
Ser	Serine
SH2	Src homology 2
SPNs	Solid-pseudopapillary neoplasms

T

TAAs	Tumor-associated antigens
TGF-α	Transforming growth-factor α
TGF-β	Transforming growth factor-β

TP53	Tumor protein p53
Tyr	Tyrosine

V

VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor-A
VNTR	Variable number tandem repeat

Resumo

O cancro do pâncreas é, presentemente, uma das áreas de estudo mais desafiantes da Oncobiologia. O diagnóstico tardio, juntamente com a falta de conhecimento do processo de carcinogénese a nível molecular, torna esta patologia uma das mais mortais em todo o mundo, sendo a taxa de mortalidade praticamente equivalente à taxa de incidência. Um maior conhecimento sobre o processo de carcinogénese pancreática que permita um diagnóstico precoce e o desenvolvimento de terapias mais eficazes é crucial.

A mucina MUC1 é uma glicoproteína transmembranar normalmente expressa na região apical das células epiteliais com uma grande relevância na proteção e lubrificação dos epitélios, na interação célula-célula e célula-matriz, assim como na sinalização celular. Nos tumores epiteliais, como o cancro do pâncreas, sabe-se que esta molécula está sobrexpressa e aberrantemente glicosilada.

O objetivo deste trabalho foi compreender o envolvimento do MUC1 na biologia das células estaminais tumorais e desvendar a contribuição das variantes de splicing alternativo para a carcinogénese do pâncreas, duas áreas científicas pouco exploradas.

A glicoproteína membranar designada de prominin-1 (CD133) foi inicialmente descrita como sendo um antigénio específico das células estaminais hematopoiéticas e células progenitoras e apesar da sua função biológica permanecer pouco conhecida é, atualmente, usada na identificação e isolamento das células estaminais tumorais em alguns tipos de cancro, incluindo o pancreático. Recorrendo a modelos *in vitro* e *in vivo* foi avaliado o envolvimento da proteína MUC1 nas vias de sinalização de uma subpopulação celular altamente tumorigénica, que expressa CD133 e se designa de $CD133^{+}$. Os nossos resultados sugerem que as células $CD133^{+}$ expressam níveis mais elevados de MUC1, contribuindo para o fenótipo tumorigénico destas células através da interação entre o domínio citoplasmático do MUC1 e a proteína β-catenina, que por sua vez modula cascatas de sinalização oncogénicas.

Apesar da maioria dos esforços até agora estarem centrados essencialmente no estudo da proteína completa do MUC1, o gene *MUC1*, tal como cerca de 95% de todos os genes humanos com mais que um exão, sofre processos de splicing alternativo. Sabe-se que este processo tem funções chave na regulação e expressão génica contribuindo para a heterogeneidade do transcriptoma e do proteoma celular. Foram identificadas até à data mais de 70 variantes de splicing do MUC1, no entanto, a relevância biológica destas isoformas continua por esclarecer. De forma a contribuir para o esclarecimento das suas funções biológicas e considerando as ferramentas disponíveis, foi usado um modelo de células pancreáticas *in vitro* com expressão da variante de splicing MUC1/S2. Foi possível observar que a expressão da isoforma MUC1/S2 é mais restrita ao citoplasma e leva a um aumento da capacidade de invasão das células geradas. Curiosamente, identificamos pela primeira vez a interação entre a isoforma MUC1/S2 e a proteína p53, o que nos pode indicar um papel desta isoforma em vias de sinalização oncogénicas. Por último, destacamos os avanços científicos

na área do MUC1 relativos aos dois principais temas enfatizados neste trabalho, salientando pontos de futuro interesse nesta área de investigação.

Abstract

Pancreatic cancer is nowadays one of the most challenging fields of the oncobiology scientific community. Late diagnosis together with the lack of a better understanding of the carcinogenic process make this pathology one of the most lethal worldwide, with almost identical incidence and mortality rates. An improvement in basic scientific knowledge of the pancreatic carcinogenesis to allow earlier diagnosis and improved therapies remain unmet needs for this disease.

MUC1 is a transmembrane glycoprotein generally expressed in the apical surface of epithelial cells with an important role in protection and lubrification of the epithelium's, in cell-cell/cell-extracellular matrix interaction and in signaling transduction. In epithelial cancers, namely in pancreatic cancer, this molecule is known to be overexpressed and aberrantly glycosylated.

The aim of this work was to understand the involvement of MUC1 in cancer stem cell biology and to disclose the contribution of MUC1 splice variants in pancreatic carcinogenesis, two unexplored scientific topics.

The pentaspan membrane glycoprotein prominin-1 (CD133) was initially described as a cell surface antigen specific of hematopoietic stem and progenitor cells and despite its biological function remaining unclear it is currently used in the identification and isolation of cancer stem cells from some malignant tumors, including pancreatic cancer. Using *in vitro* and *in vivo* models, the involvement of MUC1 in the signaling pathways of a highly tumorigenic $CD133^+$ cellular subpopulation was evaluated. The results suggest that $CD133^+$ cells have higher MUC1 expression, contributing to the tumorigenic phenotype through increased interaction between MUC1-CD and β-catenin, which in turn modulates oncogenic signaling cascades.

Although most efforts so far were focused on the full MUC1 protein, the *MUC1* gene, like more than 95% of all multiple-exon human genes, undergoes alternative splicing events. This is a key process in the regulation of gene expression, contributing to the heterogeneity of the transcriptome and the proteome. *MUC1* has more than 70 different splice variants identified yet their biological function remains to be elucidated. In order to provide insights into their biological function and taking into account the available tools, we used a pancreatic *in vitro* model transduced, using a lentivirus system, with MUC1/S2 splice variant and characterized its phenotype. It was possible to observe that MUC1/S2 expression is mostly restricted to the cytoplasm and leads to an increase in the invasive phenotype of these cells. Interestingly, an interaction between MUC1/S2 and p53 protein was identified for the first time, which can indicate a role of this isoform in signaling pathways. Finally, scientific advances in MUC1 research concerning the two topics emphasized in this work were highlighted, raising future points of interest in this research area.

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General Introduction

Cancer epidemiology

Cancer is a major concern in public health and despite the efforts to improve prevention, diagnosis and treatment, the incidence is expected to grow, mostly due to the growth and aging of the world population and the increasing prevalence of established risk factors worldwide (Torre et al. 2015). In 2012, 14.1 million new cases and 8.2 million cancer-related deaths were estimated by GLOBOCAN, through the International Agency for Research on Cancer (IARC) (Ferlay et al. 2015). Cancer epidemiology is different between developed and developing countries where lung cancer is the most incident and leading cancer death among males while breast cancer represents the most incident and lethal neoplasia among females. In developed countries, although lung cancer leads mortality rates, prostate and breast cancer are the most incident, respectively, among males and females. In Europe, prostate, breast, lung, colorectal, and stomach cancers are the most frequently diagnosed (Ferlay et al. 2013).

Pancreatic cancer

Epidemiology and risk factors

Pancreatic cancer (PC) is one of the most lethal cancers being the seventh leading cause of cancer-related deaths worldwide, for both sexes. Data from GLOBOCAN estimate that 338,000 individuals have been diagnosed with PC in 2012, being 104,000 of these new cases diagnosed in Europe. In Europe, pancreatic cancer represents the fifth leading cancer death among males and the fourth among females being 30% more common in men than in women (Ferlay et al. 2013, ACC 2014, Ferlay et al. 2015).

The major concern related with this neoplasia is that incidence rates are almost identical to mortality rates, which is reflected in a five-year survival of only 6%, and with almost no changes achieved in the last decades, it has the poorest prognosis of any major tumor type.

The incidence and mortality rates of pancreatic cancer are higher in developed countries and the numbers have been increasing over the past years, mostly due to the influence of risk factors. PC risk factors encompass demographic, lifestyle and environmental factors as well as multiple genetic syndromes. Overall, according to the National Cancer Institute (NCI), these risk factors and genetic syndromes are responsible for 30-40% of all pancreatic cancers (ACC 2014).

One of the most reliable and important risk factors in PC, as for most cancer types in adults, is age. The median age of diagnosis is 71 years old with 53% of the cases diagnosed

between 65 and 84 years old and only 3% under age 44 (ACC 2014). Ethnicity is also accountable for PC incidence/mortality being African-Americans more likely to be diagnosed with advanced disease than any other race or ethnic group (Chang et al. 2005).

Cigarette smoking is another well-established risk factor in PC development identified as a causative agent in case-control, cohort and retrospective studies (Lowenfels et al. 2001, Lynch et al. 2009, Bosetti et al. 2012). The risk of developing PC in cigarette smokers is around 2.2-fold higher and it has been estimated that 15-20 years of smoking cessation is necessary to achieve a similar risk to non-smokers (Boyle et al. 1996, Lowenfels et al. 2001, Bosetti et al. 2012). Smoking is particularly harmful in patients with *Helicobacter pylori* (HP) infection and familial pancreatic cancer (FPC) where it can result in a 2-fold and a 4-fold increased risk, respectively (Lowenfels et al. 2001, Rulyak et al. 2003, Klein et al. 2004).

In a dose-dependent relationship, heavy alcohol usage (three or more drinks per day) is also associated with a 1.22 to 1.36-fold increased risk of PC development, possibly through pancreatitis induction (Genkinger et al. 2009, Tramacere et al. 2010, Yeo and Lowenfels 2012).

Diet and obesity are other lifestyle risk factors that increase the likelihood of developing PC. Butter, saturated fats, red meat and processed food consumption increases the risk of PC independently of their role in obesity while fruit and vegetables are associated with a protective effect (Stolzenberg-Solomon et al. 2001, Larsson and Wolk 2012). Body mass index (BMI) of over 40 is associated with a relative risk of PC of 1.49 for men and 2.76 for women (Calle et al. 2003). Moreover, obesity was associated with a younger age of onset, late-stage at time of diagnosis and decreased survival rates (Stocks et al. 2009, Yuan et al. 2013). The role of obesity and diet in development of PC may include abnormal glucose metabolism, hyperinsulinemia, inflammatory changes and pancreatic hypertrophy and hypoplasia as a response to cholecystokinin-mediated lipase secretion (Gapstur et al. 2000, Stolzenberg-Solomon et al. 2005, Stattin et al. 2007, Stocks et al. 2009, Jarosz et al. 2012).

Pancreatitis can be a cause or consequence of PC and has been widely studied as a risk factor in the development of this neoplasia. Chronic pancreatitis, characterized as inflammation of the pancreas that increases over time and leads to permanent morphological changes, is considered a well-established risk factor and represents a relative risk of 13.3 for PC development. Pancreatitis occurring less than 2 years prior to cancer diagnosis is associated with a higher risk (odds ratio (OR) =13.6, 95% CI = 8.7– 22) however, it is not well established whether in this case pancreatitis precedes cancer or is a consequence of pancreatic ductal obstruction from the tumor mass (Raimondi et al. 2010, Duell et al. 2012). Chronic pancreatitis explains around 3% of all PC cases and the most common cause of chronic pancreatitis in developed countries is alcohol abuse (around 60%-90%) and less commonly hereditary and autoimmune pancreatitis (Lowenfels et al. 1999, Pandol and Raraty 2007).

Over the past 40 years, multiple studies have correlated PC and diabetes mellitus leading to the postulation that type 2 diabetes is the third most modifiable risk factor in the development of this neoplasia, after cigarette smoking and obesity (Li 2012). Type 2 diabetes represents an overall OR of 1.8 (95% CI=1.5-2.1) when compared with non-diabetics. However, this correlation is complex since long-term disease is associated with a decrease in PC relative risk when compared to PC patients that only develop diabetes 1-3 years before diagnosis (OR=2.9, 95% CI=2.1–3.9) which can indicate diabetes as an early manifestation of PC (Chari et al. 2005, Cui and Andersen 2012).

Occupational exposure to toxic and carcinogenic material such as chlorinated hydrocarbon (CHC), nickel and nickel compounds, polycyclic aromatic hydrocarbons (PAH), among others, are appointed to contribute to about 5% of all PC cases (Ojajarvi et al. 2000, Lowenfels and Maisonneuve 2004).

Despite more than 80% of all PC being due to sporadic mutations, inherited genetic changes can be an important risk factor for PC. Case-control and cohort studies demonstrated that family history can increase the risk of PC in a range of 1.9 to 13-fold, being involved in approximately 5-10% of all cases (Falk et al. 1988, Ghadirian et al. 1991, Price et al. 1996, Breast Cancer Linkage 1999, Schenk et al. 2001, Jacobs et al. 2010). Familial PC is defined when at least two first-degree relatives are affected and the relative risk is directly related with the kinship and the number of affected family members (Klein et al. 2004, Wang et al. 2007, Wang et al. 2009, Brune et al. 2010). Furthermore, familial PC is also associated to an increased risk of breast (1.6-fold, 95% CI=1.15-2.34), ovarian (2.05-fold, 95% CI=1.10-3.49) and bile duct cancer (2.89-fold, 95% CI=1.04-6.39) (Wang et al. 2009). Germline *BRCA2* mutations are the most commonly inherited genetic alterations associated with this neoplasia (Tersmette et al. 2001, Murphy et al. 2002). Different studies have found *BRCA2* mutations in 17-19% of familial PC families (Goggins et al. 1996, Breast Cancer Linkage 1999, Murphy et al. 2002, Hahn et al. 2003). Other germline mutations have also been related with an increased risk of PC such as mutations in *PALB2*, *BRCA1* and *p16/CDKN2A* genes (Moskaluk et al. 1998, Thompson et al. 2002, Rutter et al. 2004, Axilbund et al. 2009, Ferrone et al. 2009, Tischkowitz et al. 2009, Slater et al. 2010, McWilliams et al. 2011). Several germline genetic syndromes have also been associated with genetic predisposition to PC and altogether account for less than 20% of all familial PC (Murphy et al. 2002, Hruban et al. 2010). Hereditary pancreatitis, familial atypical multiple mole melanoma syndrome (FAMMM), Lynch syndrome, familial adenomatous polyposis (FAP), hereditary breast and ovarian cancer syndrome, cystic fibrosis, Peutz-Jeghers syndrome, Li-Fraumeni syndrome, ataxia-telangiectasia, Fanconi anemia and more recently Non-O blood groups are the syndromes that are documented to have an involvement in the development of this neoplasia (Giardiello et al. 1993, Goggins et al. 1996, Moskaluk et al. 1998, Breast Cancer Linkage 1999, Borg et al. 2000, Giardiello et al.

2000, Murphy et al. 2002, Hahn et al. 2003, Amundadottir et al. 2009, Kastrinos et al. 2009, Egawa et al. 2013).

The identification of risk factors, whether genetic or lifestyle related, can have a huge impact in PC incidence as a result of population behavior as well as in early diagnosis and treatment through the identification of the high-risk population that can benefit from preventive strategies.

Pancreas overview

Pancreas is a complex and highly branched gland, playing a crucial function in secretion of digestive enzymes and in glucose homeostasis. Along with the liver and biliary tract, the pancreas arises from a shared multipotent population of cells in the foregut endoderm, is located deeply in the retroperitoneum, behind the stomach, and is anatomically divided in four different parts: head, neck, body and tail. Histologically, pancreas is divided in endocrine and exocrine portions (Lack 2003, Wandzioch and Zaret 2009) (**Figure 1**).

The endocrine portion accounts for around 2% of the pancreas, encompasses the islets of Langerhans, and is responsible for secretion of hormones into the bloodstream. The Islets of Langerhans embrace different specialized cell types, each one responsible for the production of a different hormone and are densely vascularized and surrounded by acini cells. Beta (β) cells, that represent around 60% of the islet cells, are responsible for insulin production while alpha (α) cells secrete glucagon and represent around 30% of the islet cell population (Slack 1995). Somatostatin, secreted by Delta (δ) cells, is a potent inhibitor of insulin and glucagon secretion and accounts for less than 10% of the islet cells (Sakurai et al. 1974, Luft et al. 1978). Together, these three types of specialized cells are responsible for glucose homeostasis into the bloodstream by endocrine and paracrine regulation. Contrary to what happens in other animals, like rodents, recent studies showed no clustering of β cells in islets. In these studies, it was possible to observe that β cells are scattered throughout human islets with the other endocrine cells aligned along blood vessels with no particular order or arrangement (Brissova et al. 2005, Cabrera et al. 2006). Nonetheless, controversies persist about the architectural arrangement of pancreatic endocrine cells (Orci and Unger 1975, Erlandsen et al. 1976, Orci 1976, Bosco et al. 2010). Pancreatic polypeptide (PP) producing cells represent less than 5% of the islet cells and the peptides are released by vagal stimulation and, although the physiologic role remains to be clearly established, it is known to be involved in satiety sensation after food intake (Larsson et al. 1976, Asakawa et al. 1999, Batterham et al. 2003). More recently, some studies appointed the existence of a different type of cells in the endocrine pancreas, the ghrelin-producing cells, that are believed to be involved in the

control of insulin secretion, metabolic regulation and energy balance (Lee et al. 2002, Wierup et al. 2002, Wierup et al. 2004). The islets of Langerhans are distributed within the exocrine pancreas, which emphasizes its important physiological function in the modulation of the exocrine function and homeostasis (Barreto et al. 2010).

Exocrine pancreas represents nearly 95% of all pancreatic mass, being mainly composed by acinar and duct cells and has a major physiologic role in secreting enzymes that are essential for the digestive process (**Figure 1**). Acinar cells, which account for around 80% of the pancreatic exocrine cells, have a pyramidal shape that lines up in a single layer and are arranged concentrically around a lumen, giving rise to a pancreatic acinus. These cells synthesize and secrete digestive enzymes, such as Trypsinogen or Proelastase, in a catalytic inactive form into the duodenum through a complex network of ducts (Lack 2003). When these zymogens reach the gut they are activated by proteolytic cleavage (Weiss et al. 2008).

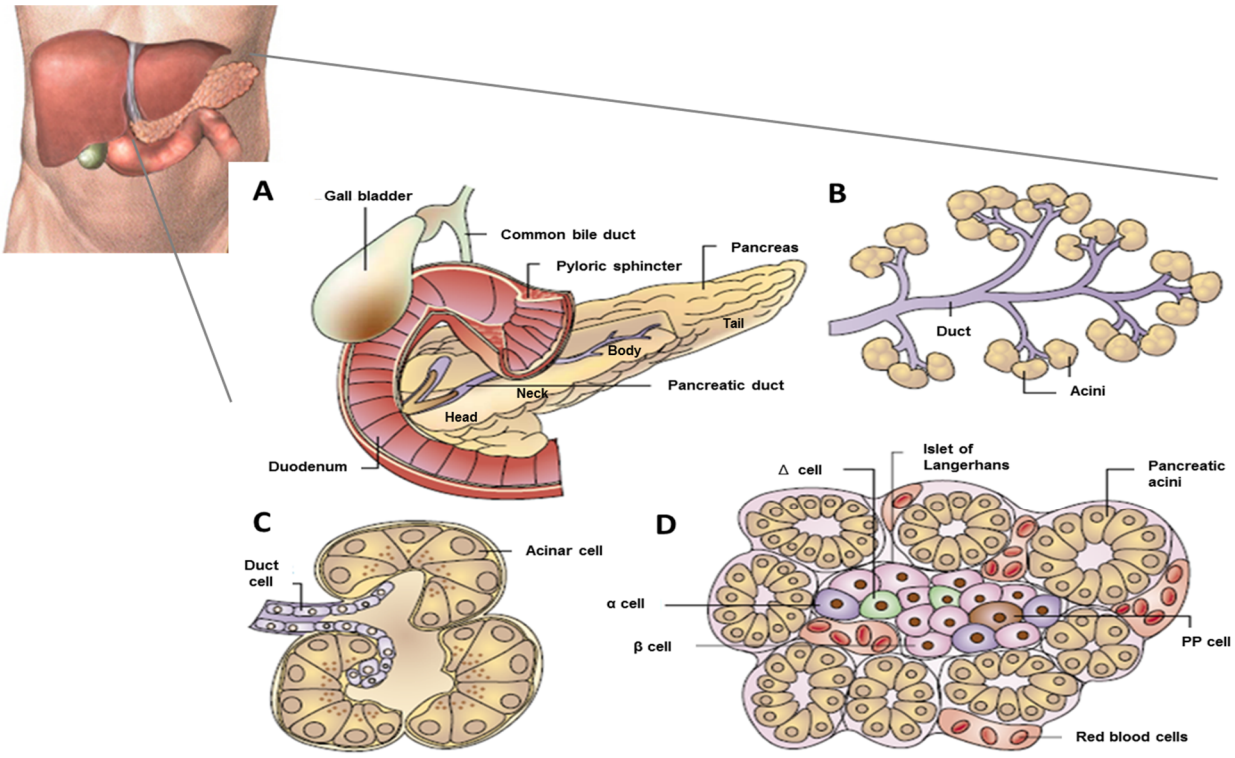

Figure 1. Sketch of pancreas anatomy. Pancreas is anatomically divided in four different parts: head, neck, body and tail **(A)**. The exocrine pancreas represents the major portion of the organ and consists of acinar and duct cells **(B)**. Individual acinus encompassing acinar cells organized in a grape-like cluster at the smallest termini of the branching duct system **(C)**. The pancreatic islet is embedded in the exocrine tissue **(D)**. [Adapted from DePinho *et al*., 2002]

Pancreatic carcinogenesis

Morphologic perspective

Different pathologies can arise in pancreas, from diabetes to several clinically and pathologically different neoplasms. These can be broadly classified into cystic or solid subtypes. The differential diagnosis of these neoplasms has a major importance in patient management and prognosis since some of them are known to be precursor lesions of PC.

The most common solid tumors that arise in the pancreas, accounting for more than 85% of all neoplasms, are adenocarcinomas. Histologically they resemble the pancreatic duct architecture, reason why they are named pancreatic ductal adenocarcinomas (PDAC) and, since they represent the majority of the invasive tumors in pancreas, they are simply called pancreatic cancer. Despite all the improvements on the biology and molecular knowledge about this pathology, PC remains practically an incurable disease with life expectation measured in months. Late diagnosis, namely in stage IIIB or IV which occurs in the majority of the patients (nearly 80-85%), contributes to a large extent for the dismal prognosis of this disease (Stathis and Moore 2010, Wolfgang et al. 2013). No early detection methodologies are available and surgery remains the unique curative treatment. However, surgical resection is only an option in pre-cancerous or early-stage (I-II) PC which constitutes only 15-20% of the patients and for those the 5-year survival rate is nevertheless 20% (Ahrendt and Pitt 2002). Late diagnosis is mostly due to the nonspecific symptoms that only appear when tumors metastasize to other organs. These symptoms may vary depending on the localization of the primary tumor, in the head, body or tail of the organ, but normally include chronic abdominal discomfort, back pain, weight loss, jaundice, occasional glucose intolerance and biliary obstruction (Koorstra et al. 2008).

It is believed that PC is a long-term developing disease being estimated that 10-30 years are required since the initiating mutation arises until manifestation of the disease with most of the somatic mutations being acquired before the development of the metastatic disease (Yachida et al. 2010). Analogous to other carcinomas, it is believed that PC results from a stepwise progression process from normal epithelium to invasive carcinoma, which was schematized, in a model proposed by Hruban and colleagues (Brat et al. 1998, Hruban et al. 2000, Koorstra et al. 2008). The most frequent precursor lesion, in around 85% of the cases, is the pancreatic intraepithelial neoplasms (PanINs). This lesion is a microscopic (<5 mm diameter) mucinous-papillary lesion that arises from the smaller epithelial ducts and is typically observed in the surrounding area of the tumor (Andea et al. 2003). PanINs are histologically divided into four different categories based on the dysplasia degree in PanIN-1A or PanIN-1B, and PanIN-2 or PanIN-3 lesions **(Figure 2)** (Hruban et al. 2000, Hruban et al. 2001, Hruban et al. 2004). These lesions have been extensively studied and with the advances in the

technology the genetic alterations associated with them are now relatively well known, which was crucial to establish PanIN as precursor lesions of PC since they share genetic alterations.

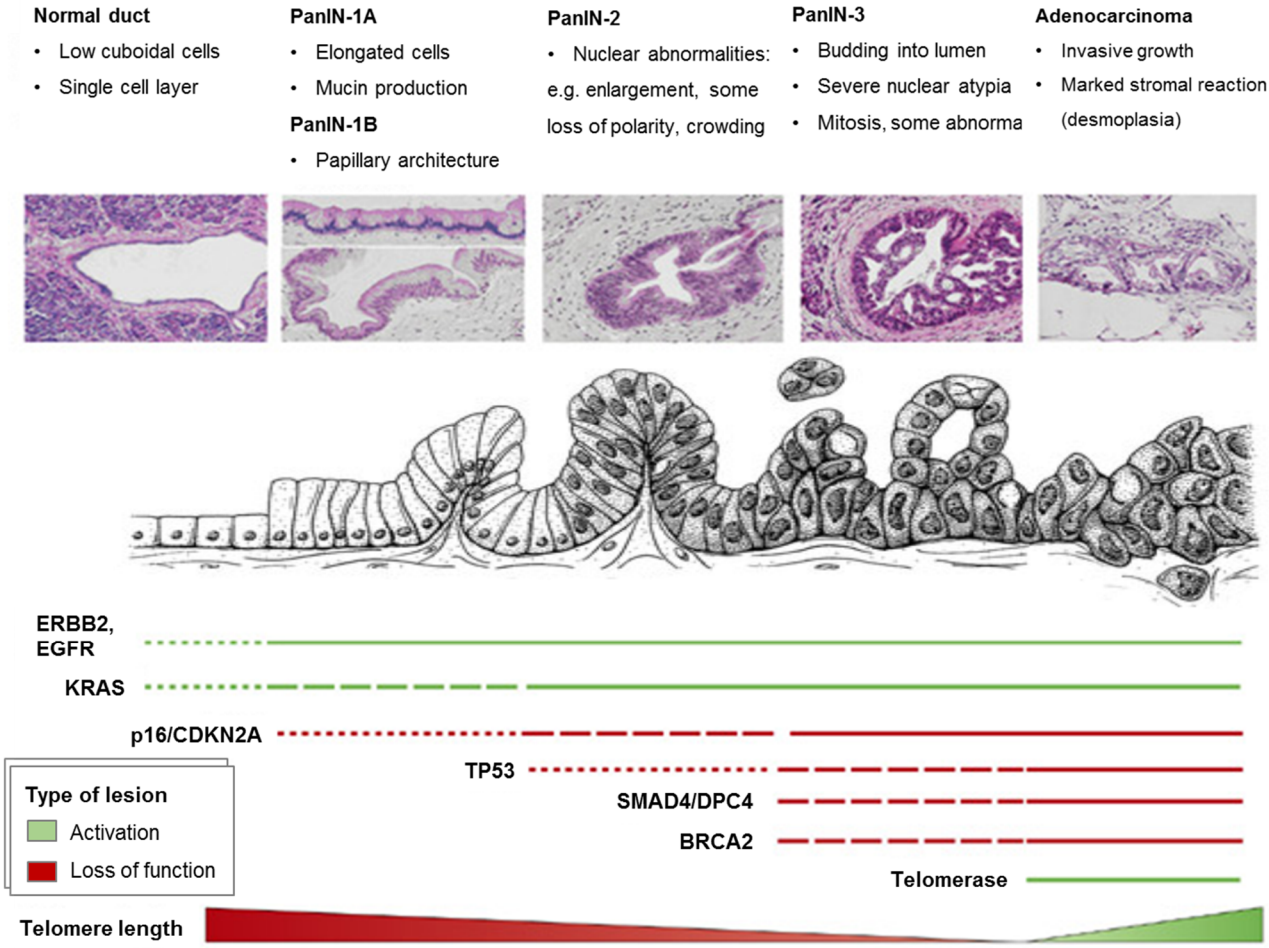

Figure 2. Progression model of pancreatic adenocarcinoma. Transformation of a normal duct epithelium into adenocarcinoma through a series of histologic and genetic alterations. The thickness of the line corresponds to the frequency of a lesion. [Adapted from DePinho *et al*., 2002; Maitra *et al*., 2003]

Some pancreatic lesions are benign and do not progress to an invasive carcinoma, however others such as intraductal papillary mucinous neoplasm (IPMN), can represent precursor lesions of invasive ductal adenocarcinoma and therefore can constitute a window of opportunity to an early diagnosis and more effective treatment. IPMNs account for around 25-30% of all pancreatic cystic lesions and are defined as mucin-producing epithelial neoplasms that can involve the major pancreatic duct, the branch ducts or both (Nagai et al. 2008). They are noninvasive and classified according to the dysplasia grade into low-grade dysplasia (adenoma), intermediate-grade dysplasia (borderline) and high-grade dysplasia (carcinoma *in situ*). Approximately one-third of the patients diagnosed with IPMNs already present with an

associated invasive carcinoma at the time of diagnosis (Niedergethmann et al. 2008, Matthaei et al. 2011, Distler et al. 2013, Zamboni et al. 2013). Histologically, based on cytoarchitectural features and immunophenotype, IPMNs can be sub-divided in intestinal, oncocytic, pancreatobiliary and gastric subtype (Furukawa et al. 2005). This histopathologic subdivision, together with the lymph node ratio and the presence or not of invasive carcinoma, plays an important role in the prognosis of these lesions and, after surgical resection of noninvasive IPMN, 5-year survival rates are reported to be at 77-100% (Niedergethmann et al. 2008, Furukawa et al. 2011, Kim et al. 2011). Another cystic lesion known as a precursor lesion of PC is the mucinous cystic neoplasm (MCN) (Zamboni et al. 2013). In contrast with IPMNs, MCNs don't establish contact with the larger pancreatic ducts and are more prevalent in women (Distler et al. 2014). Like IPMNs, they are subdivided based on the grade of dysplasia and about one-third of them become invasive (Baker et al. 2012). Nevertheless, the five-year survival of noninvasive MCN is almost 100% and even in patients that need to undergo resection due to invasive MCN the five-year survival is around 60% (Baker et al. 2012). Pancreatic neuroendocrine tumors (PanNETs) are the second most common solid type of pancreatic neoplasm and can involve any part of the pancreas. PanNETs are fully malignant tumors with a less invasive phenotype than PDACs and with a ten-year survival rate of 45% (Reid et al. 2014).

The diagnosis of pancreatic cystic neoplasms (PCNs) is increasing due to advances in cross-scan imaging technics, such as computed tomography (CT) and magnetic resonance imaging (MRI). These lesions are not uncommon, being present in around 1.9% of the patients that undergo imaging diagnosis for suspicion of pancreatic disease (Zhang et al. 2002). Furthermore, they are detected in 2.6% of the asymptomatic patients (Canto et al. 2012).

Solid-pseudopapillary neoplasms (SPNs), also known as solid and papillary tumors, papillary cystic tumors, solid cystic tumors, Frantz tumors and Hamoudi tumors are rare and almost only present in young women (Martin et al. 2002, Ng et al. 2003). Although these lesions are usually related with a good prognosis, they are malignant neoplasms with an invasive phenotype and metastasize in 10% to 15% of the patients (Tang et al. 2005, Reddy et al. 2009). Finally, serous cystic neoplasms (SCNs) are almost always benign lesions not removed by surgery unless they are large or cause symptoms and can be clinically followed (Wargo et al. 2009).

Molecular perspective

Sporadic PC results from the accumulation of acquired mutations. The earlier and most frequent point mutation in pancreatic tumorigenesis occurs in *KRAS* oncogene (Hruban et al. 1993, Moskaluk et al. 1997). Activating point mutations in this gene are present in more than 90% of the tumors making PC the neoplasia most frequently associated with mutations in the *KRAS* oncogene (Hruban et al. 1993). The majority of *KRAS* mutations occur in codon 12 of the gene being the G12D the most prevalent one (Jimeno and Hidalgo 2006, Goggins 2007, Singh and Maitra 2007, Koorstra et al. 2008). They lead to constitutively activation of the Ras protein, which is involved in cell-cycle control, triggering uncontrolled stimulatory signal to downstream signaling pathways such as RAF mitogen-activated protein kinase, phosphatidylinositol 3´-kinase (PI3K), and RalGDS pathways (Shields et al. 2000, Jimeno and Hidalgo 2006, Koorstra et al. 2008). *KRAS* mutations are found occasionally in histologically normal pancreas and in 36% of PanIN-1A, 44% of PanIN-1B, and 87% of PanIN-2/PanIN-3 lesions (Sturm et al. 1998, Luttges et al. 1999, Tascilar et al. 1999, Hingorani and Tuveson 2003, Hruban et al. 2008). Although specific KRAS downstream pathways in pancreatic carcinogenesis are not yet fully understood, there are evidences of epidermal growth factor (EGF) family signaling involvement (Korc et al. 1992, Watanabe et al. 1996, Overholser et al. 2000). Interestingly, EGF family receptors, such as epidermal growth factor receptors ErbB1 and ErbB2, are overexpressed in pancreatic carcinogenesis since low-grade PanIN lesions which points to a role of these proteins in the earliest stages of pancreatic neoplasias together with *KRAS* activation (Day et al. 1996). The screening of *KRAS* mutations is easy, however, considering that not all of the *KRAS* positive neoplasias will progress to PC the diagnostic value of these mutations alone is not very relevant. As a therapeutic target in PC, KRAS has been disappointing until now (Karp and Lancet 2007).

Mutations in tumor suppressor genes also contribute to pancreatic carcinogenesis being the most relevant mutations in the *CDKN2A*, *TP53* and *DPC4/SMAD4* genes. Inactivation of p16/*CDKN2A* tumor-suppressor gene is present in 90% of all PC. It is detected in 30% of PanIN-1, 55% of PanIN-2 and 70% of the PanIN3 lesions, indicating that it is a relatively early event in pancreatic carcinogenesis (Wilentz et al. 1998). Loss of p16/*CDKN2A* function can occur by homozygous deletion, intragenic mutation and loss of the second allele or by promoter hypermethylation (Caldas et al. 1994, Schutte et al. 1997). p16 protein is important in cell cycle G1/S checkpoint. This protein binds to CDK4 and CDK6 proteins leading to Rb-1 phosphorylation inhibition and consequent cell cycle arrest (Sherr 2000). Loss of p16 function leads to an aberrant Rb-1 phosphorylation that results in inappropriate progression through the cell cycle, decreasing apoptosis and increasing cell proliferation (Sellers et al. 1995).

Another frequent genetic alteration in pancreatic carcinogenesis is inactivation of the *TP53* tumor suppressor gene. *TP5*3 gene is the most frequently inactivated tumor-suppressor gene among all human solid tumors. This gene is inactivated in around 70% of all PC most frequently through an intragenic mutation and loss of second allele (Goggins 2007, Koorstra et al. 2008). The protein encoded by this gene is involved in a number of important biological processes, namely in the regulation of apoptosis as a response to cytotoxic stress and in DNA repair during cell cycle arrest (Kern 1994). As a result of inactivation of this protein, cell proliferation and apoptosis are deregulated contributing to tumor progression.

DPC4/SMAD4 tumor suppressor gene is also frequently inactivated during pancreatic carcinogenesis. Like *TP53*, inactivation of this gene occurs relatively late in pancreatic carcinogenesis being present in 30-40% of all PanIN-3 lesions and invasive carcinomas (Wilentz et al. 2000). *SMAD4/DPC4* encoded protein modulates the transforming growth factor-β (TGF-β) signaling pathway (Massague et al. 2000). In a normal context, TGF-β activation causes SMAD2 and SMAD3 protein phosphorylation and heterodimerizes with the SMAD4 protein. The heterodimer translocates to the nucleus where SMAD4 transactivates the transcription of specific target genes involved in cell proliferation, angiogenesis, metastasis and immune suppression (Blobe et al. 2000). This inactivation is a predictor of worse prognosis and has been shown to contribute for cell transformation in a *KRAS* mutated context (Heinmoller et al. 2000, Kojima et al. 2007).

Mutations in genes involved in DNA repair, such as *BRCA2*, are also known to contribute to pancreatic carcinogenesis in the context of a germline mutation. Germline *BRCA2* mutations are present in less than 10% invasive PC and this gene is important in repairing DNA-interstrand cross-links through homologous recombination (Goggins et al. 1996). As some chemotherapeutic agents, such as cis-platinum and mitomycin, introduce these DNA-interstrand crosslinks, tumors harboring mutations in *BRCA2* are expected to be more susceptible to treatment with these agents (van der Heijden et al. 2005). Somatic mutations (inactivation of the second allele) in *BRCA2* are found in PanIN-3 lesions but are absent in low-grade lesions, suggesting that this is a late event in PC carcinogenesis (Goggins et al. 2000).

Cytogenetic studies have shown that telomere dynamics contribute to genomic instability. Despite telomerase reactivation being necessary for immortalization of cancer cells, the telomere shortening that leads to the formation of chromosomal rearrangements by an abnormal fusion of the chromosome ends is an early event in this process (Artandi et al. 2000, Maser and DePinho 2002). In this context, the cell death that normally occurs in response to the presence of critically short telomeres, is overcomed by the inactivation of the DNA-repair machineries allowing oncogenic chromosomal alterations (Chin et al. 1999, Gisselsson et al. 2001). An analysis of a large panel of PC cell lines reveals that short telomeres and genomic instability are associated in this model (Gisselsson et al. 2000, Gisselsson et al. 2001). As

these features were observed both in low and high grade pancreatic tumors, telomere shortening is considered to be an early event in pancreatic carcinogenesis. Moreover, it was observed that telomerase activation is a late event (Kobitsu et al. 1997, Suehara et al. 1997, Gisselsson et al. 2001). More recently, it has been shown that epigenetic abnormalities and changes in mRNA expression pattern have a role in pancreatic carcinogenesis (Sato et al. 2003, Sato et al. 2006, Volinia et al. 2006, Szafranska et al. 2007).

While it is important to pursue a better understanding of the genetic signature of the precursor lesions to improve early diagnosis and treatment, it is crucial to more accurately predict which of those lesions will effectively progress to PC in order to identify which patients are indeed at risk of developing the disease and to avoid overtreatment.

Cancer Stem Cells

The cell of origin of human pancreatic cancer remains an unsolved question. Considering the ductal morphology of PC, ductal pancreatic cells have been described as the origin of these tumors. However, despite the morphological features and the expression of ductal lineage genes, the evidences supporting duct cells as the cell of origin remain weak and evidences of acinar cells as the cells of origin have been emerging (De La et al. 2008, Habbe et al. 2008, Maitra and Leach 2012). These studies, using lineage-specific transgenic expression of mutant *KRAS,* emphasize the importance of acinar to ductal metaplasia in pancreatic carcinogenesis. Despite the specific cell origin of PC not being fully elucidated it is now accepted that these cells display stemness features and is now debated if they derive from mature cells that reacquire stem cell properties during carcinogenesis or from mutated stem cells. These cells are called cancer stem cells (CSCs) or tumor-initiating cells and represent a small subpopulation of the tumor cells with self-renewal capacity and the ability to give rise to the heterogenic cellular subpopulations that are identified in tumors. This subpopulation of cells is believed to have the exclusive ability to drive tumor formation, growth, relapse and also chemoresistance and has been identified in an ever-increasing number of human tumors (Al-Hajj and Clarke 2004, Dalerba et al. 2007, Hermann et al. 2007, Li et al. 2009). Therefore CSCs have been described as potentially elective targets for therapeutic strategies (Mimeault et al. 2007). Several markers have been used to identify and isolate these CSCs from different tumor types including colon, prostate, breast and pancreas (Al-Hajj et al. 2003, Collins et al. 2005, Dalerba et al. 2007, Hermann et al. 2007, Rasheed et al. 2010). In PC, the first study was based on the expression of $CD44^+$ $CD24^+$ ESA^+ cell surface markers and showed an increase in the tumorigenic and self-renewal capacity of this subpopulation of cells, isolated from low-passage xenografts, when compared to the remaining tumor cells (Li et al. 2009). Pancreatic CSCs were also identified and isolated based on the glycoprotein prominin-1

(CD133) surface marker from pancreatic tumor samples. This subpopulation of cells revealed an exclusive tumorigenic potential, high resistance to standard chemotherapy and a close relationship with the metastatic phenotype (Hermann et al. 2007, Moriyama et al. 2010). The pentaspan membrane CD133 was initially described as a cell surface antigen specific for hematopoietic stem cells (Yin et al. 1997). Although its biological function remains unclear its use in the identification and isolation of CSCs from malignant tumors is now well established (Singh et al. 2003, Todaro et al. 2010).

A better understanding of the mechanisms behind CSC biology could potentiate the development of alternative therapies targeting this subset of cells in the expectation to overcome chemoresistance and tumor relapse.

Mucins

Mucins overview

Mucins (MUC) are large and heavily O-glycosylated proteins generally expressed in the apical surface of epithelial cells being the major compound of the mucus. They are present in relatively harsh environments like the digestive and respiratory tract and in the secretory epithelia of different organs such as the kidney, liver and pancreas (Forstner 1978). Mucin canonical functions are to provide protection from pathogens, dehydration, changes in pH and degradative enzymes. All mucins contain a proline, serine and threonine (PTS)-rich tandem repeat domain that is heavily O-glycosylated in serine and threonine residues and contributes to 50%-80% of the protein total molecular weight (Baldus et al. 2004). The specific sequence and the number of tandem repeats are variable among different mucins and individuals, due to genetic polymorphisms. N-glycosylation is also often present, although to a much lesser degree. The human MUC gene family encodes for up to 21 known proteins that can be divided in two main groups: secreted mucins and membrane-associated mucins. Secreted mucins are subdivided in two classes: gel-forming mucins (MUC2, MUC5AC, MUC5B, MUC6 and MUC19) and non-gel-forming mucins (MUC7) (Bobek et al. 1993, Gum et al. 1994, Desseyn et al. 1997, Toribara et al. 1997, Escande et al. 2001). Gel-forming mucins are typically expressed in specialized glands or goblet cells and their main function is to create the three-dimensional network of the mucus, contributing for their viscoelastic properties, through oligomerization domains in order to protect the epithelium against different injuries (Thornton and Sheehan 2004). On the other hand, non-gel-forming mucins are smaller and unable to oligomerize and are mostly secreted by salivary and lachrymal glands (Bobek et al. 1993, Gipson 2004).

Membrane-associated mucins (MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20 and MUC21), in addition to their function in

protection of the epithelia from adverse conditions, also play important biological roles in cell-cell and cell-extracellular matrix interaction and signal transduction (Carraway et al. 2003, Jonckheere and Van Seuningen 2008). They are anchored at the cell membrane through a single hydrophobic transmembrane (TM) domain and, with the exception of MUC4, all of them contain a SEA (Sea urchin sperm protein, Enterokinase and Argin) domain in the extracellular region (Jonckheere and Van Seuningen 2008).

Mucins in pancreatic carcinogenesis

The expression profile of mucins as well as the glycosylation pattern are tissue and cell type specific and can be modified with cellular differentiation state or under pathologic conditions such as tumorigenesis. In the pancreas, MUC1 and MUC6 are the predominantly expressed mucins (Balague et al. 1994, Reid et al. 1997). MUC1 is normally present at apical membranes of centroacinar cells as well as in the intralobular and interlobular ducts while MUC6 expression is mostly restricted to the pancreatic ducts. MUC5B is expressed in both acinar and ductal cells (Balague et al. 1994, Balague et al. 1995, Andrianifahanana et al. 2001). Secreted MUC2 and MUC5AC mucins and membrane-associated MUC4 mucin are absent or weakly expressed (Balague et al. 1994).

It is well known that mucin expression and the associated glycosylation pattern is altered in PC and premalignant lesions. In PanIN lesions, the most relevant precursor lesions of PC, the most altered mucins are MUC1 and MUC4. MUC1 expression increases with the progression of the PanIN lesion while MUC4 is neo-expressed early in PanIN lesions and its expression gradually increases until adenocarcinoma formation (Adsay et al. 2002, Swartz et al. 2002, Park et al. 2003). MUC5AC, like MUC4, is not normally expressed in normal pancreas being *de novo* expressed in 70% of early stage PanIN1A lesions and in 85% of PC (Kim et al. 2002). PanIN lesions also show *de novo* expression of other mucins such as MUC13, MUC15 and MUC17, which increases with disease progression to PC (Moniaux et al. 2006, Takikita et al. 2009, Haridas et al. 2011, Chauhan et al. 2012). Throughout the progression model, MUC2 expression remains unchanged (Adsay et al. 2002).

In IPMN lesions, characterization of mucin expression is used for histopathological diagnosis since intestinal, oncocytic, pancreatobiliary and gastric subtypes show a different expression pattern of these proteins (Yonezawa et al. 1999, Luttges et al. 2001, Furukawa et al. 2005, Kitazono et al. 2013). Intestinal-type IPMN lesions typically express MUC2 and MUC5AC and, more recently, a study demonstrated MUC4 expression in these lesions (Kitazono et al. 2013). On the other hand, pancreatobiliary-type lesions, normally associated with an invasive component, show MUC1 and MUC5AC expression. Oncocytic-type IPMN lesions show diffuse expression of MUC5AC and MUC6 whereas MUC1 and MUC2 show focal

positivity. Finally, common low-grade gastric-type lesions express MUC5AC and less frequently MUC6. In IPMNs, MUC1 expression is correlated with a bad prognosis while MUC5AC expression is associated with slow-growing adenomas with good prognosis. Since MCN lesions are rare, the available studies about mucin expression are scarce and a consensus has not been achieved. Luttges *et al*. showed that MUC5AC expression is present in low-grade lesions, as well as MUC2, while MUC1 is expressed in invasive MCN (Luttges et al. 2002). In contrast, Terada *et al.* reported the expression of MUC1 (7/8 cases), MUC3 (2/8 cases) and MUC5AC/MUC6 (3/8 cases) and the absence of MUC2 expression (Terada et al. 1996).

Aberrant glycosylation is a common feature among essentially all types of cancers, being more frequent than changes in the expression profile. In mucins, several tumor-associated antigens (TAAs) are found and, despite the number of biomarkers under investigation, the only biomarker in PC used in the clinical practice is based on a glycan epitope that is found in mucins. The sialylated Lewis blood group antigen or carbohydrate antigen 19-9 (CA19-9) recognizes the sialyl Lewis A oligosaccharide structure and increased levels of this antigen in the serum are correlated with tumor burden increase and poor prognosis (Magnani et al. 1983, Rhodes 1999). This biomarker is elevated in approximately 75% of PC patients with a sensitivity of 70% and a specificity of 87% (Steinberg 1990, Fong and Winter 2012). The major drawback of this biomarker is that it can be detected among patients with benign pancreaticobiliary disorders and is often normal in patients that harbor preinvasive lesions such as IPMNs and high-grade PanIN (Canto et al. 2006). Furthermore, 5-15% of the population does not even express Lewis antigens. For these reasons, CA19-9 is not recognized as a good early diagnostic tool and is mostly used in the clinical practice to monitor disease burden and response to therapy.

It is expected that recent advances in technology together with a better understanding of cell biology might lead to the identification of novel biomarkers relevant for PC early diagnosis and screening together with new targets for the development of new therapies to overcome the dismal prognosis of this disease (Melo et al. 2015, Sausen et al. 2015).

MUC1

MUC1 in cancer

As previously described, both the glycosylation and the expression of mucins are deregulated during pancreatic carcinogenesis. One of the most deregulated mucins is MUC1, reason why it will be described in more detail here.

Mucin 1 (MUC1; also known as episialin, PEM, H23Ag, EMA, MCA and CA15-3) was the first mucin to be cloned and until now it remains the best studied protein of this family. MUC1 is normally present at the apical surface of polarized epithelial cells and was also described as being present in hematopoietic and stem/progenitor cells (Agrawal et al. 1998, Brugger et al. 1999, Dent et al. 1999, Hikita et al. 2008). The *MUC1* gene is located in the long arm of chromosome 1 in position 21 (locus 1q21), and comprises seven exons ranging about 4.4kb. The mature MUC1 protein encompasses two subunits, the extracellular N-terminal subunit that comprises the signal sequence and the variable number tandem repeat (VNTR) domain, and the C-terminal subunit that comprises an extracellular stem region, a short transmembrane domain and the cytoplasmic domain (MUC1-CD) (**Figure 3**) (Gendler et al. 1990, Lan et al. 1990). The VNTR region consists of a closely identical sequence of 20 amino acids (aa) repeated 25 to 125 times. This glycoprotein is synthesized as a single polypeptide chain being autoproteolytically cleaved in the endoplasmic reticulum soon after synthesis and is present, in normal conditions, on the cell surface as a heterodimer (Parry et al. 2001, Singh and Hollingsworth 2006). Cleavage of the MUC1 precursor polypeptide occurs between glycine and serine residues of the GSVVV motif within the SEA module of the extracellular domain (Parry et al. 2001, Wreschner et al. 2002, Lillehoj et al. 2003). In the membrane, the two subunits (alpha and beta subunit) bind together through a strong non-covalent interaction (Ligtenberg et al. 1992). MUC1 is heavily O-glycosylated mostly in the VNTR region and despite N-glycosylation also being present, it is much less frequent, being the glycosylation profile of MUC1 dependent on the tissue and on the profile of glycosyltransferases expressed (Brockhausen et al. 1995, Remmers et al. 2013). MUC1, similar to other membrane-associated mucins, besides playing a central role in maintaining homeostasis and promoting cell survival in response to harsh environments, is also involved in cell signaling and transduction events mostly through MUC1-CD phosphorylation.

MUC1-CD, a highly conserved 72 aa sequence, contains several Tyr residues that represent potential docking sites for proteins with Src homology 2 (SH2) domains, such as protein kinase C (PKCδ), glycogen synthase kinase 3β (GSK-3β) and ErbB receptors like ErbB1/epidermal growth factor receptor (EGFR) (Vos et al. 1991, Li et al. 2001). Changes in the phosphorylation status of MUC1-CD can modulate its affinity for mediators of signal transduction, including β-catenin and p53 proteins (**Figure 3**) (Ren et al. 2002, Wei et al. 2007).

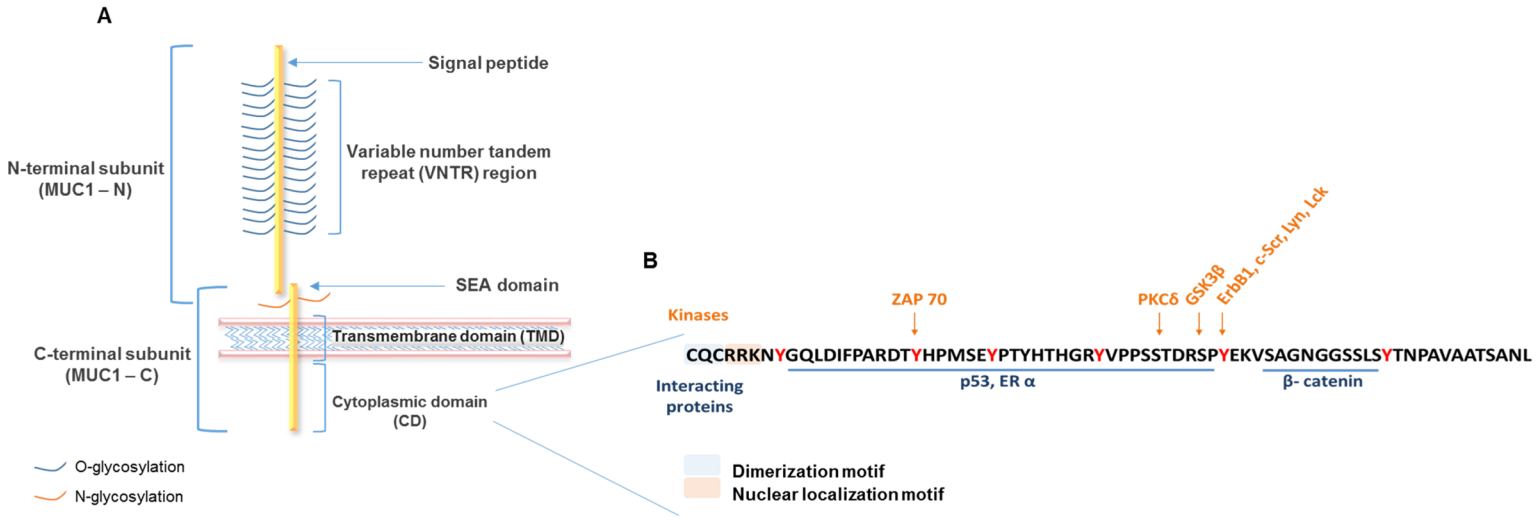

Figure 3. Depiction of MUC1 structure highlighting the cytoplasmic domain sequence. (A) N-terminal subunit and C-terminal subunit forming a stable heterodimeric complex at the cell membrane. **(B)** Amino acid sequence of MUC1 cytoplasmic domain (MUC1-CD), showing potential phosphorylation sites and protein-binding motifs.

MUC1 is overexpressed in more than 80% of the pancreatic tumors and differs from the MUC1 expressed in normal cells with respect to the biochemical features, cellular distribution and function, reason why the oncogenic role of MUC1 in cancer progression will now be reviewed. In tumors, MUC1 overexpression can be due to several mechanisms, including increased MUC1 transcription, amplification of MUC1 genomic locus or loss of post-transcriptional regulation (Merlo et al. 1989, Lagow and Carson 2002, Ahmad et al. 2009, Rajabi et al. 2010). In tumor cells, MUC1 has fewer, shorter and less branched glycans which is in contrast with normal cells that normally show more elongated and highly branched glycans (Lloyd et al. 1996). This, together with the different expression levels and cellular localization led to the establishment of MUC1 as a TAA. The hypoglycosylation of MUC1 in cancer cells impacts the stability and the subcellular localization of the protein, increasing the intracellular uptake by clathrin-mediated endocytosis. As a consequence, and since MUC1 degradation is not increased, there is an intracellular accumulation of this protein (Altschuler et al. 2000). MUC1 glycosylation has also been associated with inflammatory response since its dense glycan part entraps proinflammatory factors such as transforming growth factorα (TGF-α), interleukin 1 (IL-1) and interleukin 4 (IL-4), among others, that are released after MUC1-N shedding, inducing an inflammatory response (Cebo et al. 2001). Due to the loss of polarity in tumor cells, MUC1 is no longer restricted to the apical surface being instead spread over the entire cell surface (**Figure 4**). This loss of polarity disturbs the cell-cell and/or cell-matrix interactions, favoring the release of tumor cells into the blood circulation and moreover, enabling the interaction between MUC1 and other membrane proteins normally restricted to the basolateral domain of the cells, such as growth factor receptors (Schroeder et al. 2001). Interactions with these proteins could contribute to the growth and survival of tumor cells through activation of signaling pathways or by blocking the access of other molecules to these receptors (Hollingsworth and Swanson 2004).

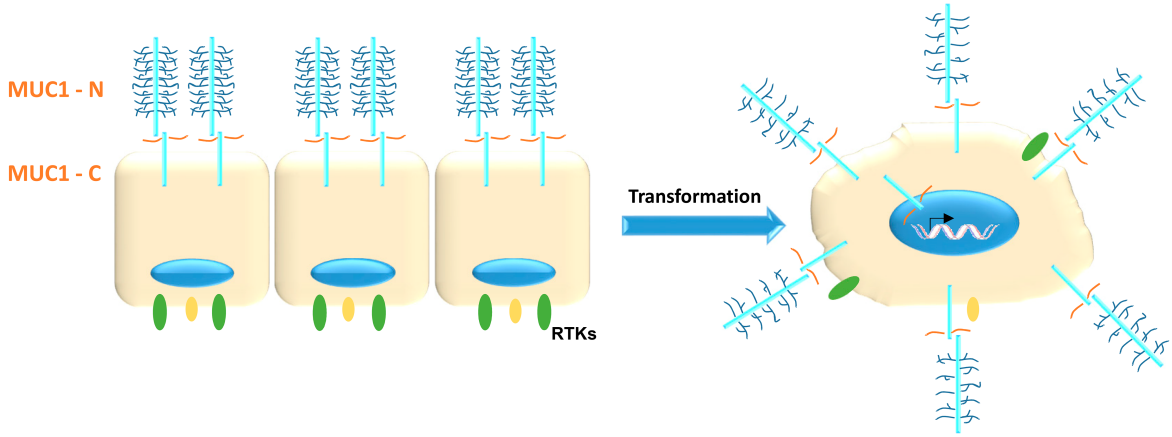

Figure 4. MUC1 in normal vs MUC1 in cancer cells. MUC1 is confined to the apical surface of normal epithelial cells. However, in tumor cells this polarity is lost and no longer restricted to the apical surface

being instead spread over the entire cell surface which potentiates the interaction between MUC1 and RTKs. These interactions could contribute to activation of signaling pathways or to block the access of other molecules to these receptors. Hypoglycosylation of MUC1 is also represented.

Although the establishment of *MUC1* as an oncogene is not consensual, its involvement in proliferation, angiogenesis, invasion and metastization is now well documented. Studies in transgenic mice and different cell lines have related MUC1 with carcinogenesis in different ways being hypothesized that, globally, MUC1 participates in the control of the local microenvironment allowing the tumor to adapt, survive and proliferate in invasive and metastatic microenvironments. The discovery that overexpression of MUC1-CD alone is sufficient to induce anchorage-independent growth and tumorigenicity of carcinoma cells *in vitro* led to a stronger focus of the research in this subunit of MUC1 (Huang et al. 2003). MUC1-CD signaling at the cell membrane is triggered by phosphorylation of the serine and threonine residues. This phosphorylation can occur in response to the activation of several surface growth factor receptors, namely, platelet-derived growth factor receptor (PDGFR) and members of the Erb family which include EGFR and ErbB2/Her2 (Riese and Stern 1998, Schroeder et al. 2001, Singh et al. 2007). Binding of the ligands to the extracellular domain of these receptors leads to the formation of a dimer that is followed by cross-phosphorylation of MUC1-CD that starts working as a docking site for the activation of different signaling pathways important in cellular proliferation, differentiation, migration and survival such as PI3K and mitogen-activated protein kinase (MAPK) pathways (Schlessinger 2000).

MUC1-CD is also a target of several different kinases including PKCδ, GSK-3β and the tyrosine kinases c-Src and Lck (Thompson et al. 2006). Phosphorylation events regulate the interaction between MUC1-CD and other intracellular binding partners such as the Wnt pathway effector or β-catenin (Yamamoto et al. 1997, Li et al. 2001, Kufe 2009). β-catenin binds to MUC1-CD through a motif similar to those present in E-cadherin and the adenomatous polyposis coli (APC), at the SXXXXXSSL sequence (Yamamoto et al. 1997). This interaction is dependent of MUC1-CD phosphorylation by c-src kinase and is inhibited by GSK-3β phosphorylation (Li et al. 2001). MUC1-CD/β-catenin complex leads to an accumulation of β-catenin in the nucleus where it acts as a transcriptional coactivator of genes controlling cellular growth, such as cyclin-D1 and c-myc, promoting tumor progression (Smalley and Dale 2001). Moreover, MUC1-CD/β-catenin interaction decreases the ability of β-catenin to interact with E-cadherin at adherens junctions leading to a decrease in cell-cell interaction (Li et al. 1998). β-catenin in the nucleus also represses E-cadherin expression and induces the transcription of epithelial-mesenchymal transition (EMT) inducers such as *Snail*, *Slug*, *Vimentin* and *Twist,* enhancing the invasive potential of tumor cells (Kalluri and Weinberg 2009, Roy et al. 2011). MUC1 and β-catenin don't interact in a normal polarized epithelium since they have different

subcellular localizations. EMT is also induced by MUC1 through regulation of miRNA involved in EMT-gene expression (Mohr et al. 2013, Rajabi et al. 2014).

Angiogenesis is another hallmark of cancer in which MUC1 plays a role. The growing of a tumor mass creates a low oxygen and nutrient microenvironment (hypoxia) that induces tumor cells to express proangiogenic factors, promoting growth of new vessels in order to adapt and survive in this environment. It has been demonstrated that MUC1 stimulates the expression of proangiogenic factors like the connective tissue growth factor (CTGF), platelet-derived growth factor subunit B (PDGF-B) and endothelial growth factor-A (VEGF-A), which are responsible for the synthesis of new blood vessels within the tumor that provide oxygen and nutrients (Kitamoto et al. 2013). In pancreatic cancer, it was also reported that MUC1 overexpression induces the synthesis and secretion of vascular endothelial growth factor (VEGF) through the AKT signaling pathway (Woo et al. 2012).

MUC1 overexpression also confers the cell survival advantages by blocking cell death mediated by both the intrinsic and extrinsic apoptosis pathways. MUC1-CD is able to bind to heat shock proteins 70 or 90 (HSP70 or HSP90). They are translocated as a complex to the mitochondria where MUC1-CD blocks the loss of mitochondrial transmembrane potential abolishing apoptosis response to oxidative, genotoxic, hypoxic and metabolic stress (Ren et al. 2004, Ren et al. 2006, Ren et al. 2006). Direct interaction between p53 and MUC1-CD was also reported in response to genotoxic stress, promoting the transcription of p53-target genes (Wei et al. 2005). A recent study showed that MUC1 overexpression in PC cells increases chemoresistance by upregulating multidrug resistance genes, in particularly, the *ABCC1* gene that encodes for the multidrug resistance protein 1 (MRP1) (Nath et al. 2013).

The evidences of MUC1 involvement in tumor progression and the reports of MUC1 peptides and small molecule inhibitors as effective inhibitors of cancer progression, both *in vitro* and *in vivo*, led to the development of MUC1-based therapies (Tinder et al. 2008, Bitler et al. 2009, Besmer et al. 2011, Raina et al. 2011). However, all of them are relatively recent and clinical trials are still ongoing in different models, namely, multiple myeloma, breast, prostate, colorectal and pancreatic tumors, in order to understand the potential of these targeted therapies.

MUC1 splice variants

Alternative splicing is a key process in the regulation of gene expression contributing to the heterogeneity of the transcriptome and proteome and is estimated to occur in over 95% of all multiple-exon human genes. This mechanism generates multiple mRNAs from a single gene during pre-mRNA maturation and results in a variety of different proteins, that differ in their amino acid sequence. Alternative splicing has been associated with a large number of

diseases, including cancer (Wang et al. 2008). *MUC1* has alternative isoforms generated by different splicing pathways, including exon skipping, full or partial intron retention and alternative 5′ and 3′ splice sites (Zhang et al. 2013) The best characterized MUC1 isoform is the polymorphic mucin-like type 1 transmembrane protein, MUC1/REP or MUC1/TM, which represents the full molecule. MUC1/SEC, a secreted truncated isoform, retains the VNTR region and is devoided of the transmembrane and cytoplasmic region, being mostly associated with absence of malignancy. This isoform was identified as being secreted by several different cells, such as breast and colon cells, benign ovarian tumors, in the sera of breast cancer patients and also in human milk (Smorodinsky et al. 1996, Xing et al. 2001, Obermair et al. 2002, Hey et al. 2003). Although more than 70 isoforms have been identified, the full length sequence has only been reported for some.

Recently, 78 different MUC1 isoforms were isolated from human cervical and breast cancer cell lines and from human activated T cells, being MUC1/A, MUC1/B, MUC1/C, MUC1/D, MUC1/X, MUC1/Y and MUC1/ZD the most abundant ones (Zhang et al. 2013). This study identified exon 2, which contains the VNTR region, as the most skipped exon among all different isoforms using the same 5' splice site and variable 3' splice sites. The authors point several different reasons for this to occur, namely, the large size of this exon which can be as high as 6,200bp; the presence of core splicing signals in the VNTR sequence, such as the 5' splice site, the 3' splice site and the branch point site; the presence of cis-regulatory elements at the junction of the repeats that have been described as intron splice enhancers, or the existence of Nova splicing factor binding sites (YCAY, with Y being any pyrimidine) in each repeat, which can result in a blockage of exon inclusion when bound by Nova. On the other hand, the MUC1-CD region remains unchanged in almost all MUC1 short isoforms, allowing it to keep the important signaling function (Zhang et al. 2013).

MUC1/A, MUC1/B, MUC1/C and MUC1/D encode a "full-length" MUC1 having a fixed splice donor site near the 5´ end of intron 1 and variable splice acceptor sites near the 3´ and 5´ end of intron 1 and exon 2, respectively. MUC1/A retains 27bp of intron 1 without causing a reading frameshift while MUC1/B has intron 1 completely excised. MUC1/C and MUC1/D show excision of portions of exon 2, 9bp and 35bp, respectively, keeping the reading frame (Oosterkamp et al. 1997, Obermair et al. 2001). MUC1/A and MUC1/B are differentially expressed in prostate, cervical, ovarian and breast cancer (Obermair et al. 2002, Schmid et al. 2003, Strawbridge et al. 2008).

MUC1/Y, MUC1/X and MUC1/ZD splice variants arise from a fixed splice donor at the 5´end of exon 2 and different splice acceptor sites around the 3′ end of exon 2 resulting in MUC1 isoforms that lack the VNTR region but retain the transmembrane and cytoplasmic domain. MUC1/Y, an uncleaved splice variant, doesn't exhibit mucin-like features altough it is expressed by diverse human secretory epithelial tumors, being undetectable in the adjacent

normal tissue and was shown to be involved in tumor initiation and progression *in vivo*, in breast cancer (Zrihan-Licht et al. 1994, Baruch et al. 1999, Hartman et al. 1999, Levitin et al. 2005). It was described that MUC1/Y undergoes tyrosine/serine phosphorylation being potentially able to interact with proteins containing a SH2 domain, such as Grb2, triggering a cell signaling cascade and working in a similar way to cytokine receptors. (Zrihan-Licht et al. 1994, Baruch et al. 1999). MUC1/SEC is capable of binding to MUC1/Y in a region homologous to the ligand binding sites of cytokine receptors, inducing its phosphorylation and stimulating cell signaling pathways (Baruch et al. 1999). MUC1/Y was also shown to be overexpressed in ovarian and prostate cancer and associated with breast cancer (Baruch et al. 1997, Hanisch and Muller 2000, Schut et al. 2003)

MUC1/X and MUC1/ZD were also associated with malignant ovarian tumors (Obermair et al. 2002). MUC1/X, like MUC1/Y, misses the VNTR region but has a different acceptor site 18 aa upstream to MUC1/Y. It is highly expressed in cervical and ovarian cancer cells and contrary to MUC1/Y, this isoform undergoes proteolytic cleavage (Baruch et al. 1997, Levitin et al. 2005, Levitin et al. 2005, Kumari and Sudandiradoss 2013). MUC1/ZD also lacks the VNTR region and contains a unique C-terminal sequence of 43 aa resulting from a shift in the reading frame (Levitin et al. 2005).

More recently, a truncated genomic fragment of human MUC1 was shown to induce epithelial to mesenchymal transition in mammary mouse cells (Horn et al. 2009). In addition, a MUC1 transmembrane cleaved form (MUC1*) was reported to have an important role in chemoresistance to standard chemotherapy agents (Fessler et al. 2009) as well as to be an accurate marker of pluripotency in human embryonic stem cells (Hikita et al. 2008). This isoform contains only 45 aa of the MUC1 extracellular domain, lacking the VNTR region.

Currently, there is a lack of clear understanding about the expression and the biological function of all these splice variants mostly due to the absence of specific antibodies.

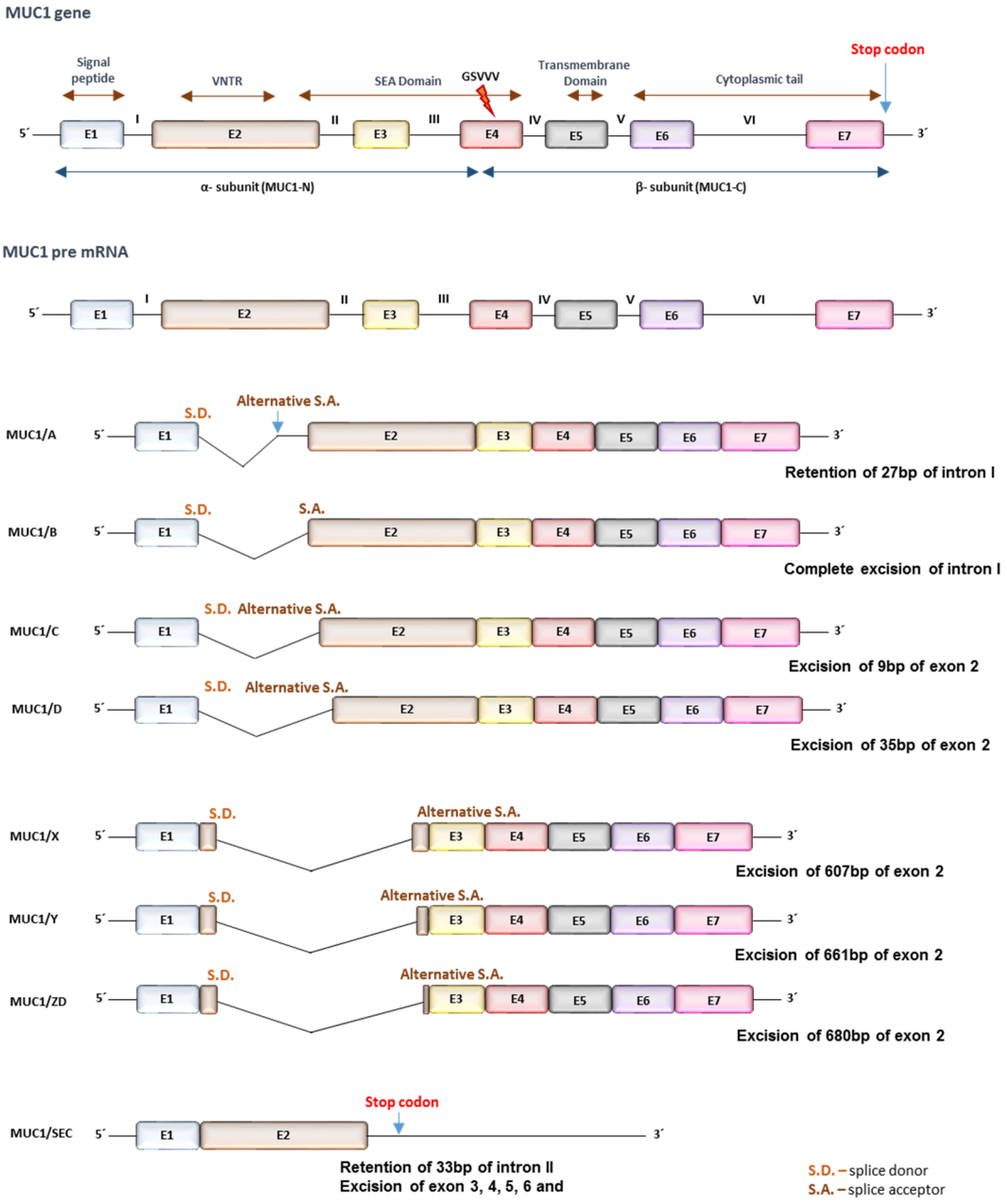

Figure 5. Schematic representation of the MUC1 gene and the different isoforms. (A) The MUC1 gene comprises seven exons (E1 to E7 colored boxes) and six introns (I to VI). Exons encoding the corresponding domains are indicated by an arrow. (B) MUC1 pre-mRNA is spliced into four main variants of mature MUC1 mRNA – MUC1/A, MUC1/B, MUC1/C, and MUC1/D. (C) MUC1/X (or MUC1/Z), MUC1/Y, and MUC1/ZD splice variants, which completely lack the VNTR region as well as the truncated MUC1/SEC isoform (adapted from Nath *et al.*, 2014).

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Chapter 2

Aim and Objectives

Pancreatic cancer remains one of the deadliest human malignancies despite the scientific and clinical efforts in the field. This panorama is mainly due to an absence of consistent symptomatology in the early stages of the disease and the lack of more accurate diagnosis techniques and molecular markers that together impair an early diagnose. Surgery remains the unique hope of cure and, even when possible, the success of this approach is limited while other treatment options do not bring a remarkable survival improve. Therefore, it is urgent to better understand the biology and molecular background of this disease to anticipate the diagnosis of individuals with pancreatic cancer and to develop new target therapies.

Since MUC1 cloning in 1990, a lot has been learnt on its role in tumor promotion, progression and relapse and its association with pancreatic cancer is now well established. Nevertheless, several questions remain to be answered such as the involvement of MUC1 in the stem phenotype of the pancreatic CSC subpopulation and the relevance of alternative splice variants of this gene in the pancreatic carcinogenesis.

Taking these questions into account, the general aim of the present work was to determine the involvement of MUC1 in the CSC phenotype and to investigate the biological function of MUC1 splice variants in pancreatic carcinogenesis.

Specific objectives

1. **Evaluate the involvement of MUC1 in pancreatic CSCs phenotype;**

In the first part of the work we evaluated the involvement of MUC1 and its signaling partners in the phenotype of pancreatic tumor-initiating cells expressing the CD133 surface marker. We used magnetic cell sorting (MACs) to select a population enriched in CD133 expressing cells from a pancreatic cell line. The stemness potential of the generated cells was validated through a tumorigenic assay.

The expression profile of MUC1 and putative signaling partners was assessed in this subpopulation of cells to evaluate the involvement of MUC1-mediated oncogenic signaling cascades in pancreatic tumor-initiating cells.

2. **Evaluate the involvement of MUC1 splice variants in the phenotype of pancreatic tumors;**

In the second part of the work we evaluated the impact of MUC1/S2 splice variant in the pancreatic tumor phenotype and oncogenic signaling. We overexpressed, in a pancreatic tumor cell line, the MUC1/S2 splice variant and characterized its sub-cellular localization.

Proliferation, invasion, migration and MUC1-p53 interaction was also evaluated in the generated cells.

3. **Highlight scientific advancements in MUC1 research with a special emphasis in cell differentiation and MUC1 splice variants raising future points of interest in this research area.**

Chapter 3

Evaluation of MUC1 involvement in pancreatic CSCs phenotype

Effect of MUC1/β-catenin interaction on the tumorigenic capacity of pancreatic CD133^{+} cells

Effect of MUC1/β-catenin interaction on the tumorigenic capacity of pancreatic CD133^{+} cells

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Abstract

Despite the fact that the biological function of cluster of differentiation (CD)133 remains unclear, this glycoprotein is currently used in the identification and isolation of tumor-initiating cells from certain malignant tumors, including pancreatic cancer. In the present study, the involvement of mucin 1 (MUC1) in the signaling pathways of a highly tumorigenic $CD133^+$ cellular subpopulation sorted from the pancreatic cancer cell line HPAF-II was evaluated. The expression of MUC1-cytoplasmic domain (MUC1-CD) and oncogenic signaling transducers (epidermal growth factor receptor, protein kinase C delta, glycogen synthase kinase 3 beta and growth factor receptor-bound protein 2), as well as the association between MUC1 and β-catenin, were characterized in HPAF-II $CD133^+$ and $CD133^{low}$ cell subpopulations and in tumor xenografts generated from these cells. Compared with HPAF $CD133^{low}$ cells, HPAF-II $CD133^+$ cancer cells exhibited increased tumorigenic potential in immunocompromised mice, which was associated with overexpression of MUC1 and with the accordingly altered expression profile of MUC1-associated signaling partners. Additionally, MUC1-CD/β-catenin interactions were increased both in the HPAF-II $CD133^+$ cell subpopulation and derived tumor xenografts compared with HPAF $CD133^{low}$ cells. These results suggest that, in comparison with HPAF $CD133^{low}$ cells, $CD133^+$ cells exhibit higher expression of MUC1, which contributes to their tumorigenic phenotype through increased interaction between MUC1-CD and β-catenin, which in turn modulates oncogenic signaling cascades.

Introduction

The pentaspan membrane glycoprotein prominin-1, also known as cluster of differentiation (CD)133, was initially described as a cell surface antigen specific for hematopoietic stem cells and progenitor cells (1,2). The biological function of CD133 remains unclear. However, it is currently used in the identification and isolation of tumor-initiating cells from certain malignant tumors, whereby it correlates with poor prognosis (3–5). Tumor-initiating cells, also called cancer stem cells (CSCs), are characterized by their self-renewal capacity and the ability to generate cell subpopulations during tumor growth (6–8). Although CD133 is a reasonable marker of various CSCs, several types of cancer arise from cells with different markers (9,10).

Pancreatic cancer is the fifth most lethal cancer in developed countries, with a 5-year survival rate of <6% (11). Early metastasis, late diagnosis and the low effectiveness of the currently available therapies contribute to its high mortality rate (12). A previous study on

pancreatic cancer identified that cells expressing CD44, CD24 and epithelial-specific antigen surface markers were associated with an increase in the tumorigenic and self-renewal capacity of tumor cells isolated from primary tumors or low-passage tumor xenografts (8). However, these markers do not identify CSCs within all pancreatic tumors, and other studies revealed that the use of CD133 to isolate tumor-initiating cells yielded populations of cells with enhanced tumorigenic potential, high resistance to standard chemotherapy and a close association with metastatic phenotype (7,13). In addition, a recent study confirmed that enforced expression of CD133 enhanced the aggressive behavior of pancreatic cancer cells (14).

MUC1 is a heavily glycosylated transmembrane glycoprotein expressed at low levels in the apical surfaces of epithelial cells (15). This glycoprotein possesses oncogenic properties, and is overexpressed in >80% of pancreatic tumors, contributing to tumor progression, metastasis and mortality in patients with pancreatic cancer (16–20). The MUC1 gene encodes a protein comprised of a large extracellular domain with a tandem repeat region, a transmembrane domain and a highly conserved cytoplasmic domain (MUC1-CD), which participates in several oncogenic signaling pathways (21). MUC1-CD is highly conserved, and contains seven tyrosine residues and several serine and threonine residues that represent potential docking sites for proteins with Src homology 2 domains and recognition sites for receptor tyrosine kinases and other kinases, including protein kinase C delta (PKCδ), glycogen synthase kinase 3 beta (GSK3β) and ErbB receptors such as epidermal growth factor receptor (EGFR) (22). Furthermore, MUC1-CD contains a serine-rich motif that functions as a β-catenin binding site, and the phosphorylation of MUC1-CD modulates this affinity (23). MUC1-CD/β-catenin interactions enhance the malignant phenotype of tumor cells by regulating the activity of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors, thus modulating the expression of several genes involved in the tumorigenic phenotype, including target genes in the Wnt signaling pathway (24). Recently, a transmembrane cleaved form of MUC1 has been reported to exert an important role in chemoresistance to standard chemotherapy agents (25), and to potentially serve as an accurate marker of pluripotency in human embryonic stem cells (26). The expression of MUC1 in CSCs has been documented by a novel antibody against tumor-associated MUC1 that recognizes a sequence in the tandem repeat region of MUC1, which is different from the sequences recognized by the majority of commercially available antibodies against MUC1 (27).

Based on the reported associations of MUC1 with CSCs, the present study aimed to investigate the potential contribution of MUC1 to the oncogenic signaling pathways of $CD133^+$ pancreatic cancer cells. The results revealed that MUC1/β-catenin interactions are associated with enhanced tumorigenic properties of $CD133^+$ pancreatic cancer cells.

Material and methods

Cell culture

The human pancreatic cell line HPAF-II was obtained from the American Type Culture Collection (Manassas, VA, USA), and was cultured in RPMI 1640 medium Gibco; Thermo Fisher Scientific Inc.,Waltham, MA USA containing GlutaMAXTMI (Gibco; Thermo Fisher Scientific, Inc.) and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 50 mg/ml gentamicin (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were grown at 37°C with 5% CO_2 in a humidified atmosphere.

CD133 cell-surface expression analysis by flow cytometry

The expression levels of CD133 in the HPAF-II cell line were assessed by flow cytometry with an anti-CD133/2-phycoerythrin (PE) monoclonal antibody (MAb) [#130-080-901; mouse immunoglobulin (IgG)1; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany]. A mouse IgG1 MAb served as a control (#130-092-212; Miltenyi Biotec GmbH).

To perform flow cytometry analysis, cells were trypsinized when 80% of confluence was reached. For each analysis, 5×10^5 cells were used. Cells were incubated with a mouse IgG1 MAb solution (1:80) for 10 min at 4°C, and next resuspended in an anti-CD133/2-PE antibody solution (1:10) for 10 min at 4°C in the dark. Upon incubation, the cells were washed with 0.1% PBS two times, and resuspended in 500 µl magnetic-activated cell sorting (MACS) buffer [phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA)], prior to be analyzed in a FACSCalibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). A cell suspension that was only incubated with mouse IgG1 MAb was used as a control. Analysis of the results was performed using FlowJo version 7.2.5 software (FlowJo, LLC, Ashland, OR, USA).

MACs

Cell subpopulations ($CD133^-$ and $CD133^+$) were isolated using a MACS system and microbeads coupled to anti-CD133/1 MAb (Miltenyi Biotec GmbH).

Magnetic separation was performed using the MidiMACSTM magnetic separation kit (Miltenyi Biotec GmbH), according to the manufacturer's protocol with minor alterations. Briefly, 1×10^8 cells were washed twice with 0.1% PBS and passed through a pre-separation filter (30 µm) in order to remove cell clumps. Subsequently, the cell suspension was incubated with human IgG FcR Blocking Reagent (1:3 in MACS buffer; Miltenyi Biotec, GmbH) and CD133 microbeads (1:5; Miltenyi Biotec, GmbH) for 30 min at 4°C. Following the incubation step, cells

were washed twice with 0.1% PBS, and the pellet was resuspended in 500 µl MACS buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA). The cell suspension was then transferred to an LS column (Milteny Biotec GmbH) previously hydrated with 3 ml buffer, and placed in a magnetic support. The total effluent was collected as the $CD133^-$ fraction, and the column was then washed three times with 3 ml buffer. Next, the column was removed from the magnet and, with the aid of a plunger, 5 ml MACS buffer were used to flush the microbeads-labeled cells out of the column. The effluent was collected as the $CD133^+$ fraction.

The $CD133^-$ cell subpopulation was subsequently passed through the LS column, and washed three times with 1 ml MACS buffer to further deplete the remaining $CD133^+$ cells. Both $CD133^+$ and $CD133^-$ fractions were centrifuged (300 × g; Centrifuge 5810R; Eppendorf, Hamburg, Germany), and the pellets were resuspended in culture medium and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

In vivo tumorigenic assay

The present in vivo tumorigenic assay was approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center (Omaha, USA; protocol 98-088-03FC). Three groups of non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (n=5/group) were subcutaneously injected in the right dorsal flanks with 3,500 HPAF-II cells [wild-type (wt), $CD133^{low}$ or $CD133^+$]. Mice were bred and maintained under pathogen-free conditions, which included: A 12 h light/12 h dark cycle, 6 AM/6 PM; water bag accessible at all times; Nestlets (Animal Specialties and Provisions, LLC, Quakertown, PA, USA) or NestPaks (WF Fisher and Son, Inc., Somerville, NJ, USA) for enrichment; 18–23°C with 40–60% humidity; and Standard Chow food, similar to LabDiet 5010 (protein 23%; fat content not less than 4.5%). Animals were observed twice a day by trained veterinary staff and once a day by laboratory staff from the Eppley Institute for Research in Cancer and Allied Disease (Omaha, NE, USA). Mice were euthanized 4 weeks following cell injection, which was the time point when it was necessary to euthanize the first mouse due to the initial signs of suffering. The maximum tumor size achieved was 263.8 mm3. Animals were sacrificed with the aid of CO_2. Following 5 min without signs of heartbeat or respiration, the animals were subjected to cervical dislocation to ensure mortality. Tumors were collected, fixed in 10% formalin (Thermo Fisher Scientific Inc.) and embedded in paraffin (Thermo Fisher Scientific Inc.) prior to sectioning (Shandon™ Finesse™ 325 microtome; Thermo Fisher Scientific Inc.). Growth of internal tumors was evaluated by direct examination (palpation), or by careful observation of animal behavior and estimation of post-procedure pain, discomfort, distress or morbidity. Anesthesia, when required, was induced by intraperitoneal administration of ketamine hydrochloride (100 mg/ml; injectable-RL 3760; NDC-0409-2051-05; Hospira, Inc.,

Lake Forest, IL, USA) and xylazine hydrochloride (20 mg/ml; injectable-AnaSed NADA; 139–236; Lloyd, Inc., Shenandoah, IA, USA).

Immunohistochemistry (IHC)

Tumor xenografts were paraffin-embedded and sectioned at 4-µm thickness. IHC staining to detect CD133 protein expression in tumor xenografts was performed using the Dako EnVision System (Dako, Glostrup, Denmark). Antigen retrieval was performed in an IHC-Tek™ Epitope Retrieval Steamer Set (IHC World, LLC, Woodstock, MD, USA) for 40 min with 10 mM citrate buffer pH 6.0 (Thermo Fisher Scientific Inc.), following deparaffinization in xylene (Thermo Fisher Scientific Inc.) and rehydration. The slides were cooled for 20 min at room temperature, and endogenous peroxidase was blocked with 3% H_2O_2 (Merck Millipore, Darmstadt, Germany) for 5 min. Primary antibody incubation was performed for 1 h at room temperature with a mouse anti-human CD133/1 MAb (1:25; clone AC133; Miltenyi Biotech GmbH). Slides were next washed in Tris-buffered saline with Tween 20 (Grisp, Porto, Portugal), and incubated with Dako REAL EnVision-horseradish peroxidase (HRP) secondary antibody (Dako) for 30 min at room temperature. For signal detection, the slides were incubated for 5 min with 3,3′-diaminobenzidine chromogen (Dako). Next, tissues were counterstained with hematoxylin (Richard-Allan Scientific™; Thermo Fisher Scientific Inc.) for 3 min, dehydrated, cleared, mounted with Histomount medium (Richard-Allan Scientific™; Thermo Fisher Scientific Inc.) and cover slipped. Hematoxylin and eosin (Richard-Allan Scientific™; Thermo Fisher Scientific Inc.) staining was performed upon antigen retrieval following a standard protocol (28).

Protein extraction and Western blot analysis

The expression levels of MUC1-CD and oncogenic signaling proteins were evaluated by western blotting. Unsorted HPAF-II cells and sorted $CD133^{low}$ and $CD133^{+}$ cell subpopulations were cultured to 80–90% confluence. Upon washing twice with PBS, lysis buffer [10 mM Tris pH 7.4, 150 mM NaCl, 0.1% (v/v) sodium dodecyl sulfate (SDS; Bio-Rad Laboratories, Inc., Hercules, CA, USA), 1 mM phenylmethylsulfonyl fluoride and 1% (v/v) Triton X-100] was added, and cells were scraped. Cell lysates were incubated on ice for 1 h and centrifuged (14,000 × g; Centrifuge 5417R; Eppendorf) for 30 min at 4°C to collect the supernatants. Protein content was assessed with a bicinchoninic acid protein assay kit (Bio-Rad Laboratories, Inc.), as described in the manufacturer's protocol.

Protein extracts were analyzed by 10% SDS-polyacrylamide gel electrophoresis (Invitrogen; Thermo Fisher Scientific, Inc.), transferred to a polyvinylidene fluoride membrane (GE Healthcare Life Sciences, Chalfont, UK) and incubated overnight at 4°C with anti-MUC-1

Armenian hamster MAb (1:300; catalogue no. Ab-5; Thermo Fisher Scientific, Inc.), anti-EGFR mouse MAb (1:200; catalogue no. sc-81449; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-PKCδ rabbit MAb (1:200; catalogue no. sc-213; Santa Cruz Biotechnology, Inc.), anti-GSK3β mouse MAb (1:200; catalogue no. sc-53931; Santa Cruz Biotechnology), anti-growth factor receptor-bound protein 2 (GRB2) mouse MAb (1:200; catalogue no. sc-8034; Santa Cruz Biotechnology, Inc.), anti-β-catenin MAb (1:1,000; catalogue no., 610153; BD Biosciences) and anti-β-actin MAb (1:2,000; catalogue no. sc-69879; Santa Cruz Biotechnology, Inc.) in 5% non-fat milk diluted in PBS containing 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA). Next, membranes were washed three times with PBS containing 0.1% Tween 20, and incubated with the corresponding goat anti-Armenian hamster (catalogue no. sc-2443), anti-mouse (catalogue no. sc-2005) or anti-rabbit (catalogue no. sc-2004) peroxidase conjugated antibody (1:2,000; Santa Cruz Biotechnology, Inc.) in 5% non-fat milk diluted in PBS containing 0.1% Tween 20. Proteins were visualized using an enhanced chemiluminescence detection kit (Bio-Rad Laboratories, Inc.).

Immunoprecipitation assay

The interaction between MUC1-CD and β-catenin in the HPAF-II cell line was evaluated by immunoprecipitation. Proteins from cell lysates (750 µg) were incubated overnight at 4°C with protein G-agarose beads (Sigma-Aldrich) previously linked to anti-MUC1 Ab-5 MAb and normal Armenian hamster IgG (eBioscience, Inc., San Diego, CA, USA). Following three washes, the immune complexes were dissociated from the beads with reducing NuPAGE® buffer (Invitrogen; Thermo Fisher Scientific, Inc.). The immunoprecipitates and cell lysates were separated in 12% Tris-glycine gels (Invitrogen; Thermo Fisher Scientific, Inc.), and immunoblotted following the aforementioned procedure.

***In situ* Proximity Ligation Assay (PLA)**

PLA was used to assess the close proximity (and putative interaction) between MUC1-CD and β-catenin in tumor xenografts. PLAs were performed using Duolink® In Situ Detection Reagents Brightfield (Olink Bioscience, Uppsala, Sweden), according to the manufacturer's protocol. Antigen retrieval was performed in an IHC-Tek™ Epitope Retrieval Steamer Set for 40 min with 10 mM citrate buffer pH 6.0, following deparaffinization and rehydration. Subsequently, the slides were incubated at 37°C for 30 min with a blocking solution (Olink Bioscience) in a humidity chamber.

For β-catenin staining, the mouse primary antibody was used under the same conditions as the ones above described for IHC, and a secondary anti-mouse antibody conjugated with Duolink® In Situ PLA® Probe Anti-Mouse MINUS (Olink Bioscience) was added, followed by 1-h incubation at 37°C in a humidity chamber.

For MUC1 staining, anti-MUC1 Ab-5 primary antibody directly conjugated with DuolinkII Probemarker Plus (Olink Bioscience) was used. The conjugation of the antibody with the probe was performed following the manufacturer's protocol, and hybridization was conducted for 1 h at 37°C in a humidity chamber. Following the ligation of the probes for 30 min at 37°C, amplification of the signal was performed for 120 min at 37°C; both steps occurred in a humidity chamber. To detect the signal, the slides were incubated with HRP-labeled probes and chromogen (catalogue no., DUO92012-30RXN; Olink Bioscience). Subsequently, tissues were counterstained with hematoxylin, dehydrated, cleared and mounted with Histomount medium.

Results

Isolation of a $CD133^+$ cell subpopulation from the HPAF-II cell line

Low-passage/highly tumorigenic samples of the HPAF-II cell line (10^4 cells produced tumors in 100% of animals; data not shown) were evaluated for CD133 expression levels by flow cytometry. The results indicated that low-passage HPAF-II cells contained ~4% $CD133^+$ cells (Fig. 1A). These cells were isolated using MACS, and both $CD133^+$ and $CD133^-$ subpopulations were cultured. To evaluate the enrichment obtained with the sorting methodology used, CD133 was again measured by flow cytometry in the above two cell subpopulations prior to injection into immunodeficient mice (**Fig. 1B**). The results revealed that the $CD133^+$ subpopulation was highly enriched in $CD133^+$ cells. However, the putative $CD133^-$ subpopulation retained a very low percentage of cells expressing CD133, and was therefore called $CD133^{low}$. Repeated selection did not improve the performance of this procedure (data not shown).

The sorted cells were evaluated for tumorigenicity and tumor phenotype. The $CD133^+$ enriched subpopulation exhibited increased tumorigenic potential when injected subcutaneously into NOD/SCID mice, since higher number of tumors were obtained from these cells (**Table I**), and tumor growth was initiated at earlier time points (3 weeks), compared with the $CD133^{low}$ population (**Table I**). IHC analysis demonstrated that the xenografts recapitulated the HPAF-II CD133 subpopulation expression levels. Tumors derived from the HPAF-II $CD133^+$ subpopulation retained high CD133 expression levels, with limited negative cells, while the HPAF-II $CD133^{low}$ xenografts were largely negative for expression of CD133. The parental HPAF-II wt-derived xenografts displayed a small percentage of $CD133^+$ cells, similar to the original cell line (**Fig. 1C**).

Table I. *In vivo* tumorigenic assay.

	Time (weeks)[a]			
Cell Subpopulation	**1**	**2**	**3**	**4**
HPAF II WT	0/5	0/5	0/5	1/5
HPAF II CD133^{low}	0/5	0/5	0/5	1/5
HPAF II CD133^{+}	0/5	0/5	2/5	4/5

[a] Data represent the number of animals with tumors vs. the total number of animals injected with different cell subpopulations. **CD**, cluster of differentiation; **wt**, wild-type.

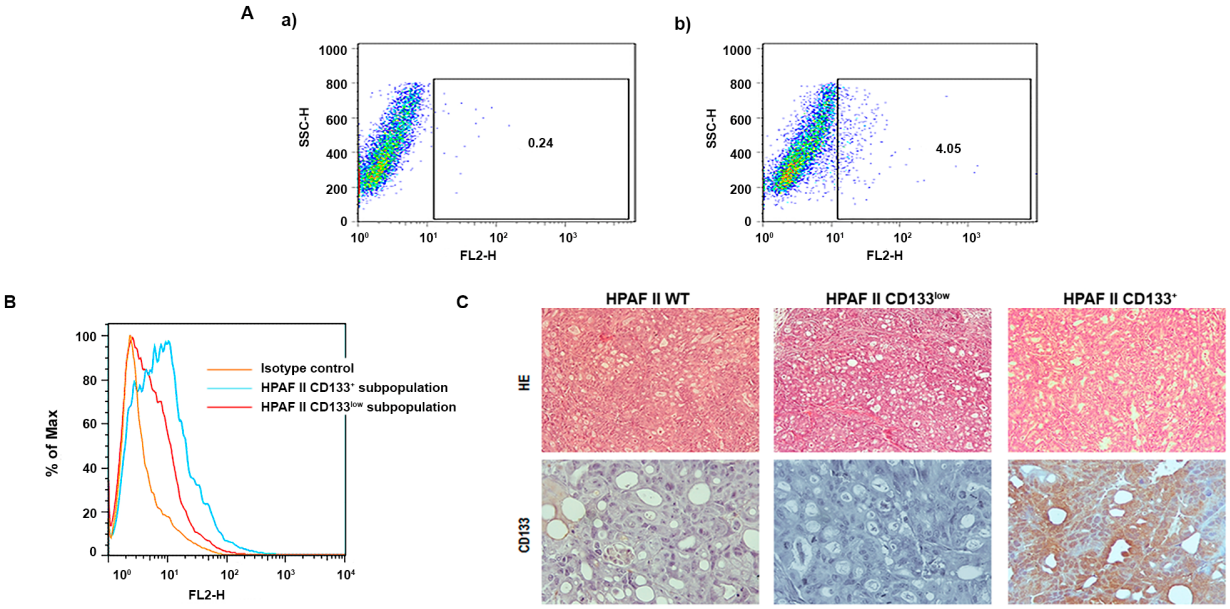

Figure 1. Validation of the CSC model. (A) Identification of a CSC subpopulation (CD133^{+} cells) in the HPAF-II pancreatic cancer cell line and evaluation of CD133 expression in cell subpopulations sorted by flow cytometry. **(a)** Isotype stained cells were used as controls. **(b)** HPAF-II cells stained with CD133/2-phycoerythrin monoclonal antibody. **(B)** Enrichment of HPAF-II CD133^{+} subpopulation isolated by magnetic-activated cell sorting represented on a frequency distribution histogram**.** The HPAF-II CD133^{+} subpopulation exhibits 8.89% of CD133^{+} cells, while the HPAF-II CD133^{low} subpopulation exhibited 3.07% of CD133^{+} cells, representing an enriched and a depleted population, respectively. **(C)** CD133 expression in HPAF-II tumor xenografts determined by immunohistochemistry (magnification, x400). Hemotoxylin and eosin staining was used to reveal the morphology of the tumors (magnification, x100). CSC, cancer stem cell; CD, cluster of differentiation; H&E, hematoxylin and eosin; SSC-H, measures cell granularity or internal complexity; FH2, phycoerythrin detection; % of Max, % of maximum (normalization method).

Expression of MUC1 in the CD133^{+} cell subpopulation

In order to evaluate the relevance of MUC1 glycoprotein in CD133^{+} cell biology, the expression levels of MUC1 were analyzed in CD133^{low} and CD133^{+} subpopulations by immunoblotting (**Fig. 2A**). HPAF-II CD133^{+} cells were highly enriched in MUC1 expression, compared with HPAF-II wt and HPAF-II CD133^{low} cells. In addition, MUC1 expression levels in the HPAF-II CD133^{low} cell subpopulation were lower than in HPAF-II wt cells (**Fig. 2A**).

Expression of MUC1 signaling partners in CD133^{+} cells

MUC1 function in oncogenic pathways depends on the phosphorylation of its CD by several kinases, including EGFR, PKCδ and GSK3β (21). Since it is not possible to assess the phosphorylation status of MUC1-CD due to the unavailability of antibodies sensitive to phosphorylation, the expression of selected kinases in CD133^{+} cells was evaluated by immunoblot analysis. The results indicated that CD133^{+} cells were enriched in EGFR and PKCδ expression, whereas CD133^{low} cells were enriched in GSK3β expression. The protein expression levels of GRB2 were equivalent in HPAF-II wt, HPAF-II CD133^{+} and HPAF-II CD133^{low} cells (**Fig. 2B**).

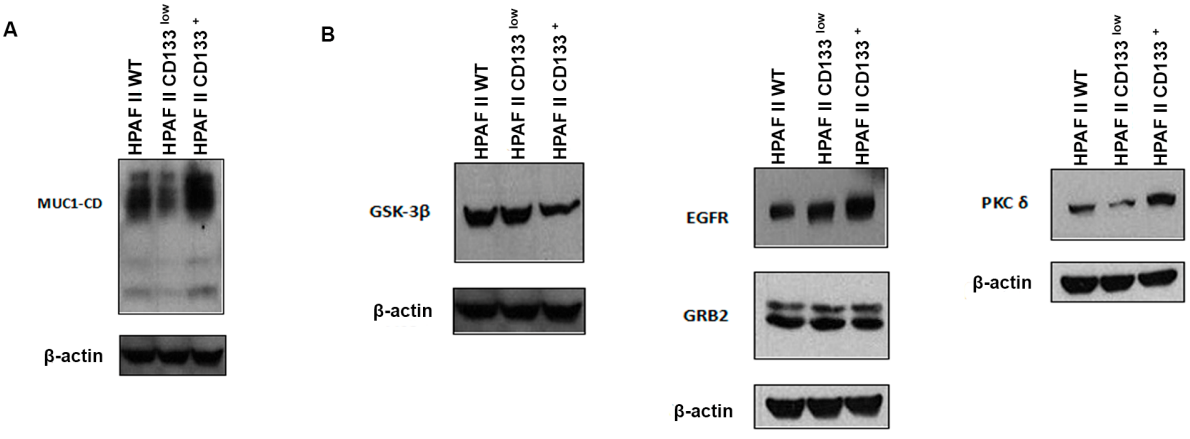

Figure 2. Expression of MUC1 and signaling partners. (A) Expression of MUC1 in HPAF-II wt, HPAF-II CD133^{low} and HPAF-II CD133^{+} cells was evaluated by western blotting. β-actin was used as a loading control. **(B)** Expression of MUC1 signaling partners (epidermal growth factor receptor, growth factor receptor-bound protein 2, protein kinase C delta and glycogen synthase kinase 3 beta) in HPAF-II wt, HPAF-II CD133^{low} and HPAF-II CD133^{+} cells was evaluated by western blotting. β-actin was used as a loading control. MUC1, mucin 1; MUC1-CD, mucin 1 cytoplasmic domain; EGFR, epidermal growth factor receptor; PKCδ, protein kinase C delta; GSK3β, glycogen synthase kinase 3 beta; GRB2, growth factor receptor-bound protein 2; CD, cluster of differentiation; wt, wild-type.

MUC1 and β-catenin interaction in the HPAF-II cell line and tumor xenografts

MUC1-CD contains docking sites for β-catenin, and the interactions between MUC1 and β-catenin are known to contribute to the malignant phenotype of tumor cells by modifying the expression of target genes in the Wnt signaling pathway (24,29). To assess if MUC1/β-catenin interaction was potentiated in CD133^{+} cells, MUC1 was immunoprecipitated from cell lysates of HPAF-II wt, HPAF-II CD133^{+} and HPAF-II CD133^{low} cells, followed by β-catenin immunoblotting. An enrichment in MUC1/β-catenin interaction was observed in HPAF-II CD133^{+} cells. β-catenin expression levels were similar in all conditions (**Fig. 3A**).

MUC1/β-catenin interactions in tumor xenografts were confirmed by in situ PLA. Significant interactions between MUC1 and β-catenin were observed in HPAF-II wt and HPAF-II CD133^{+}-derived xenografts, but almost no interactions were observed in HPAF-II CD133^{low}-derived tumors. The most abundant interactions were observed in the CD133^{+} tumors (**Fig. 3B**). In all cases, the interaction signals were predominantly observed in the nuclei of the cells.

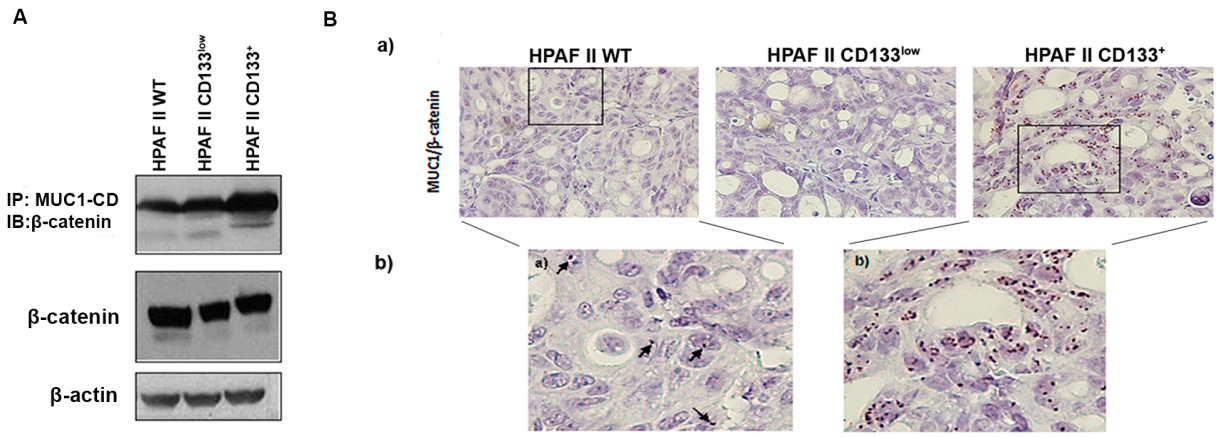

Figure 3. Evaluation of MUC1/β-catenin interaction in HPAF-II cells and xenografts. (A) β-catenin expression and its interaction with MUC1-CD in HPAF-II wt, HPAF-II CD133^{low} and HPAF-II CD133^{+} cells was evaluated by immunoprecipitation and western blot analysis. β-actin was used as a loading control. **(B)** In situ PLA in tumor xenografts was used to evaluate the interaction between MUC1 and β-catenin; magnification, **(a)** x400; **(b)** x650 (Duolink in situ Detection Reagents Brightfield staining). Brown dots correspond to proximity/interaction between MUC1 and β-catenin. Arrows indicate the PLA signals in HPAF-II wt tumors. IP, immunoprecipitation; IB, immunoblotting; CD, cluster of differentiation; wt, wild-type; MUC1, mucin 1; MUC1-CD, mucin 1 cytoplasmic domain; PLA, proximity ligation assay.

Discussion

In the present study, the involvement of CD133 and MUC1 in the highly tumorigenic low-passage pancreatic cancer cell line HPAF-II, which was derived from the ascites of a pancreatic cancer patient, was investigated (30). Using a well-established CD133 selection method, an isolated $CD133^+$ cell subpopulation was demonstrated to exhibit features associated with CSCs (enhanced tumorigenicity) and concomitant enriched expression of MUC1.

CSCs are known to aberrantly activate canonical signaling pathways (31–33). Recently, a MUC1 spliced form was reported to be associated with the differentiation status of stem cells (34). In the present study, the expression of MUC1 and oncogenic signaling transducers (EGFR, PKCδ, GSK3β and GRB2), as well as the MUC1/β-catenin association, were characterized in pancreatic cancer cells that expressed CD133. MUC1-CD, EGFR and PKCδ expression levels were increased in the HPAF-II $CD133^+$ subpopulation, while GSK3β expression was decreased, and no significant differences were observed regarding GRB2 and β-catenin expression levels. These results clearly demonstrate that pancreatic HPAF-II $CD133^+$ cells have a distinct expression profile, which includes MUC1 and its associated signaling partners, compared with the subpopulation of cells that do not express the stem cell surface marker CD133.

MUC1-CD contains docking sites for molecules such as β-catenin, and the association of these proteins is modulated by motifs that may be phosphorylated by several kinases, namely EGFR, PKCδ, GSK3β and GRB2 (35). The phosphorylation of MUC1-CD influences its interaction with β-catenin, which directly binds at the MUC1-CD motif SAGNGGSSLS (22,23). In the present study, increased interactions between MUC1-CD and β-catenin were observed in the HPAF-II $CD133^+$ subpopulation, which was correlated with enhanced expression of EGFR and PKCδ, and decreased expression of GSK3β (24,36–42). It is known that MUC1-CD phosphorylation by EGFR and PKCδ promotes interactions between β-catenin and MUC1, while phosphorylation by GSK3β leads to a decrease in this association (39–41). It was observed in the present study that EGFR and PKCδ expression were upregulated, while GSK3β expression was downregulated, in the HPAF-II $CD133^+$ subpopulation, compared with the HPAF-II $CD133^{low}$ subpopulation, which likely explains the observed increase in MUC1-CD/β-catenin interactions in the $CD133^+$ subpopulation, despite the fact that the steady-state levels of β-catenin remained unchanged in these cells.

The interactions between MUC1-CD/β-catenin influence several tumorigenic processes. Binding of MUC1-CD to β-catenin suppresses the capacity of β-catenin to interact with E-cadherin at adherens junctions, resulting in the loss of cell-cell adhesion, thus playing a

relevant role in tumor invasion (43). The MUC1-CD/β-catenin complex contributes to β-catenin stabilization by blocking its GSK3β-mediated phosphorylation and consequently its degradation in the proteasome (24). Furthermore, MUC1-CD/β-catenin is translocated to the nucleus, where it may enhance the activity of β-catenin in association with TCF/LEF transcription factors, thus promoting cell proliferation and survival through upregulation of the transcription of Wnt target genes (36–38,42). Recently, this process has been associated with a metastatic gene expression signature and an epithelial-to-mesenchymal transition phenotype of tumor cells (44). In the present study, the results of in situ PLA for tumor xenografts revealed that $CD133^+$ tumors exhibited frequent MUC1-CD/β-catenin interactions, with the MUC1-CD/β-catenin complex being mainly present in the cellular nuclei, where it presumably binds to transcription factors and activates the transcription of genes involved in cell proliferation and survival.

In summary, the present study has demonstrated for the first time that pancreatic $CD133^+$ cells display enhanced expression of MUC1 and its associated signaling partners. CD133 and MUC1 expression are associated with an aggressive tumor phenotype, partly through the production of enhanced MUC1-CD/β-catenin interactions, and this may partly explain the behavior of pancreatic CSCs.

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Chapter 4

Evaluation of MUC1 isoforms involvement in pancreatic tumors phenotype

MUC1/S2 splice variant in pancreatic carcinogenesis

MUC1/S2 splice variant in pancreatic carcinogenesis

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Abstract

MUC1 is a heavily glycosylated transmembrane glycoprotein normally expressed at low levels in the apical surface of epithelial cells and overexpressed in more than 80% of pancreatic tumors. MUC1 overexpression is well known to be correlated with tumor initiation, tumor progression and poor survival of cancer patients. The *MUC1* gene encodes a protein with a large extracellular domain with a tandem repeat region, a self-cleaving domain, a transmembrane domain and a highly conserved cytoplasmic domain (MUC1-CD) that participates in several oncogenic signaling pathways. MUC1 isoforms, generated by alternative splicing events, have been differentially associated with carcinogenic processes and their functional significance remains largely unknown.

The main objective of this work was to study the relevance of the MUC1/S2 splice variant in pancreatic carcinogenesis using an *in vitro* model based on a transformed pancreatic cell line, transduced using a lentivirus system, with MUC1/S2 splice variant and MOCK construct. To achieve this goal, we evaluated the impact of the expression of this splice variant in proliferation, migration and invasion of the generated cells. Moreover, we evaluated the subcellular localization of MUC1/S2 and its interaction with p53 protein. It was possible to observe that MUC1/S2 expression is mostly restricted to the cytoplasm and leads to an increase in the invasive phenotype of these cells. Interestingly, we identified for the first time an interaction between MUC1/S2 and p53 protein, which can indicate a role of this isoform in signaling pathways. No changes were detected in proliferation and migration of the transduced cells. We validated the use of our model to study MUC1 splice variants and provided some insights to disclosure MUC1/S2 biological function in pancreatic carcinogenesis.

Introduction

Mucin 1 (MUC1) is a transmembrane glycoprotein heavily O-glycosylated and expressed in the apical surface of epithelial cells of the mammary gland, prostate, stomach and pancreas, among others, providing protection to the epithelium from pathogens colonization, dehydration, changes in pH and degradative enzymes (1-3). The mature MUC1 protein encompass two subunits, the extracellular N-terminal subunit that comprises the signal sequence and the variable number tandem repeat (VNTR) domain, and the C-terminal subunit that comprises an extracellular stem region, a short transmembrane domain and the cytoplasmic domain (MUC1-CD) (4-6). This glycoprotein is synthesized as a single polypeptide chain being autoproteolytically cleaved in the endoplasmic reticulum soon after synthesis and is present, in normal conditions, on the cell surface as a heterodimer (7, 8). The cleavage of MUC1

precursor polypeptide occurs between the glycine and serine residue of the GSVVV motif within the SEA (sea urchin sperm protein, enterokinase, and agrin) module of the extracellular domain (8-10). In the membrane, the two subunits bind together by a strong non-covalent interaction (4). MUC1 is heavily O-glycosylated mostly in the VNTR region and despite N-glycosylation also being present it is much less frequent. The glycosylation profile of MUC1 is dependent on the tissue and on the glycosyltransferases expressed in the tissue (11, 12). MUC1, together with other mucins, plays a central role in maintaining homeostasis and promoting cell survival in response to harsh environments. In normal conditions MUC1 functions as a cell-surface receptor and sensor, triggering cell signaling responses to external environment through post-translational modification of the cytoplasmic tail.

MUC1 is overexpressed and aberrantly glycosylated in a diversity of epithelial cancers, being correlated with tumor initiation, tumor progression and worse outcome of cancer patients (13-15). Pancreatic cancer (PC) is one of the deadliest cancer-related diseases worldwide with almost no changes in survival rates being achieved in the last decades and MUC1 is overexpressed in more than 80% of these tumors.

Most efforts in MUC1 research have focused on the full MUC1 protein and its role in cancer initiation and progression. However, MUC1 undergoes alternative splicing events that give rise to different isoforms which can play different biological roles.

Alternative splicing is a key process in the regulation of gene expression contributing to the heterogeneity of the transcriptome and proteome and is estimated to occur in over 95% of all multiple-exon human genes. This mechanism generates multiple mRNAs from a single gene during pre-mRNA maturation and results in a variety of different proteins that differ in their aa sequence. Alternative splicing has been associated with a large number of diseases, including cancer (26). *MUC1* gene comprises seven exons and six introns and a variety of isoforms can be generated by alternative splicing, intron retention and exon skipping. The best characterized MUC1 isoform is the polymorphic mucin-like type 1 transmembrane protein, MUC1/REP or MUC1/TM, which represents the full molecule. Although more than 70 isoforms have been identified so far, the full-length sequence has only been reported for some. MUC1/SEC, a secreted truncated isoform, retains the VNTR region and is devoided of the transmembrane and cytoplasmic regions being mostly associated with absence of malignancy. This isoform was identified as being secreted by several different cells, like benign ovarian tumors, and is present in the sera of breast cancer patients and in human milk (27, 28).

Recently, 78 different MUC1 isoforms were isolated from human cervical (HeLa) and breast cancer (MCF7) cell lines, T-cell leukemia Jurkat cells and from human activated T cells, being MUC1/A, MUC1/B, MUC1/C, MUC1/D, MUC1/X, MUC1/Y and MUC1/ZD the more abundant ones (29). This study identified exon 2, which contains the VNTR region, as the most skipped exon among all different isoforms using the same 5’ splice site and variable 3’ splice sites. On

the other hand, MUC1-CD region remains unchanged in almost all MUC1 short isoforms, allowing them to keep the important signaling function (29).

Different splice variants are involved in epithelial to mesenchymal transition (EMT) induction, resistance to standard chemotherapy agents as well as being described as accurate markers of pluripotency in human embryonic stem cells (6, 30, 31).

MUC1/S2 isoform was identified in human cervical (HeLa) and breast cancer (MCF7) cell lines and is absent in T-cell leukemia Jurkat cells and human activated T cells (29). This splice variant has a retention of 27bp of intron I and excision of 659bp from exon 2 and total excision of exons 3, 4 and 5, lacking the transmembrane domain and cleavage site of the SEA domain. Despite a few reports showing the involvement of MUC1 isoforms in cell biology, the functional significance of each of these spliced variants is not yet well understood.

In this work we intended to disclosure the role of MUC1/S2 splice variants in the phenotype of pancreatic cancer.

Material and Methods

Cellular model and culture conditions

The cell line model used in this work is based on the hTERT-THPNE cell line (THPNE), a transformed cell line derived from a non-tumorigenic epithelial hTERT-HPNE cell line isolated from a normal pancreatic duct and immortalized with the catalytic subunit of telomerase (32, 33). THPNE cell line was generated, as described by Campbell and colleagues, by sequential retroviral mutant K-Ras (G12D), E6/E7, and SV40 small t antigen transduction in order to mimic some of the oncogenic modifications present in the currently accepted pancreatic cancer progression model (32, 34). Afterwards, we transduced THPNE cell line with MUC1/S2 splice variant cDNA sequence (AY327584) based on pLVX-EF1α-IRES-Puro vector (Clontech). The control was performed using an empty pLVX-EF1α-IRES-Puro vector (MOCK). The efficiency of the transduction was confirmed by Western blot and immunofluorescence.

To establish a MUC1/S2 stable clone we performed limited dilutions from a transduced cell population. Generated cell lines were cultured in 75% low-glucose Dulbecco´s Modified Eagle Medium (DMEM) (Gibco) supplemented with 25% M3F medium (INCELL Corporation LLC, TX), fetal bovine serum (FBS) (Gibco) at a final concentration of 5%, 50 mg/ml gentamicin (Invitrogen) and 10ng/ml human EGF. Cells were grown at 37ºC with 5% CO_2 in a humidified atmosphere.

Lentivirus production and transduction

MUC1/S2 splice variant and MOCK vector were co-transfected into 293FT (Life Technologies) cell line using ViraPower Packaging Mix (Life Technologies) according to manufacturer's recommendations. 293FT cell line was grown in DMEM supplemented with 10% FBS and 50 mg/ml gentamicin. After 24 and 72 hours, lentivirus particles were harvested and THPNE cell line incubated with the respective lentivirus particles containing the MUC1/S2 splice variant and MOCK. After overnight incubation, media with the lentivirus was removed and fresh media was added.

Protein extraction and Western blot

Expression levels of MUC1 were evaluated by western blot. After washing cells twice with PBS, lysis buffer (10mM Tris pH 7.4, 150mM NaCl, 0.1% (p/v) SDS, 1mM PMSF, 1% (v/v) Triton X-100) was added and cells were scraped. Lysates were incubated on ice for 1 hour and centrifuged for 30 minutes at 4ºC to collect the supernatants. Protein content was assessed by the BCA Protein Assay Kit (BioRad), according to the manufacturer's recommendations.

Protein extracts were analyzed in a 10% SDS-PAGE (Invitrogen), transferred to a PVDF membrane (Amersham Biosciences) and blotted overnight at 4ºC with MUC-1 Ab-5 Mab (diluted 1:300, NeoMarkers). After three washes with PBS-0.1%Tween 20, goat anti-hamster peroxidase conjugated antibody (1:2000, DAKO) in 5% non-fat milk (PBS-0.1%Tween20) was added. Proteins were visualized using an enhanced chemiluminescence detection kit (BioRad).

Immunofluorescence

To assess transduction efficiency, immunofluorescence was performed by culturing cells in plastic coverslips until confluency. After this step, they were washed once in PBS-1% and fixed for 15 minutes at room temperature (RT) in 4% paraformaldehyde and 120mM sucrose. The cells were then incubated for 15 minutes with 0.1M glycine in order to neutralize residual paraformaldehyde and permeabilized for 15 minutes with 1% BSA/0.1% Triton X-100 in PBS. After three washes in PBS, the primary antibody, MUC-1 Ab-5 Mab, against MUC1 cytoplasmic tail (that is present in MUC1/S2 isoform) was incubated for 1 hour at RT. Cells were washed in PBS and incubated with DyLight^{TM} 488-conjugated AfiniPure Goat anti-armenian hamster (Jackson ImmunoResearch) for one hour in the dark at RT. After washing, slides were mounted in Vectashield/DAPI (Vectorlabs) and images acquired by confocal microscopy.

For the MUC1/S2 isoform specific organelle localization study the same immunofluorescence protocol was used and the primary antibodies against proteins specific

for each organelle were incubated together with MUC-1 Ab-5 Mab overnight at 4ºC. The antibodies used were: anti-COX IV protein for the mitochondria (1:300,3E11, Cell Signaling), anti-Calveolin for the Endoplasmic reticulum (1:300, Caveolin-2 (D4A6), Cell Signaling), Anti-Golgi Complex for the Golgi apparatus (1:300, Anti-Golgi Complex (ab103439), abcam) and anti-LAMP1 protein for the Lysosomes (1:200, LAMP1 (D2D11), Cell Signaling). MUC1 secondary antibody used was DyLight™ 488-conjugated AfiniPure Goat anti-armenian hamster (green color) and Alexa Fluor® 594-conjugated Goat anti-rabbit was used for specific cell compartments (red color). Co-localization levels (shown as yellow spots) were evaluated as a percentage of co-localization in a specific compartment compared with overall expression levels (100%) of MUC1/S2 isoform in the cell.

Brdu assay

In order to evaluate cell proliferation, we performed bromodeoxyuridine (Brdu) incorporation assay according to manufacturer's recommendations. Briefly, $1.0x10^4$ cells/well for stable clones were seeded in coverslips on 6-well plates (four replicates each) for 72 hours. Cells were incubated at 37ºC with 5% CO_2 in a humidified atmosphere and after the incubation period the medium was removed and cells incubated with 10μM BrdU for 1 hour under the same conditions. Afterwards, a standard immunofluorescence protocol using a primary antibody anti-BrdU (Clone Bu20a; Dako) was performed. The number of BrdU incorporated cells was determined on a fluorescence microscope.

Wound healing assay

In order to address differences in cell migration we seeded $20x10^4$ cells/well in 6-well plates and a wound was performed 12 hours after seeding. Cells were imaged every 10 min at 37°C in an atmosphere of 5% CO_2, 95% air for 9 hours by time-lapse microscopy. Measurements of wound closure were calculated as $Distance\ t_0 - Distance\ t_{9h}$.

Matrigel invasion assay

Cell invasion was assessed using BD Biocoat™ Matrigel™ invasion chambers, 8μm PET membrane (BD Biosciences). Cells were seeded in triplicate at $3.0x10^4$ cells/insert (24-well plates) in FBS free medium and 20% FBS containing medium was added to the bottom of the growth well, as an attractant. After 22 hours incubated at 37ºC with 5% CO_2 in a humidified atmosphere, non-invading cells were removed with the help of a cotton swab and the invading cells fixed in methanol for 15 minutes. Membranes were removed from the insert and mounted in a slide with Vectashield/DAPI. Invading cells were counted under a fluorescence microscope. Data is expressed as a ratio between invasive cells in control and clone.

In situ Proximity Ligation Assay (PLA)

Proximity ligation assay was used to assess the close proximity (and possible interaction) between MUC1-CD and p53 using the DuoLink II fluorescence kit (Olink®Bioscience, Sweden) according to the manufacturer's instructions. Cells were grown in slides until confluent, fixed in methanol for 15 minutes, permeabilized for another 15 minutes and incubated overnight at 4 °C with MUC-1 Ab-5 Mab (diluted 1:200, NeoMarkers), and/or anti-p53 Ab-2 Mab (diluted 1:100, Santa Cruz Biotechnologies). Slides were washed with PLA wash buffer and incubated with PLA probe anti-mouse MINUS and anti-hamster directly conjugated with PLA probe Plus (previously labeled using Duolink II Probemarker Plus (OLink Bioscience), according to the manufacturer's instructions), followed by a 1 hour incubation at 37ºC in a humidity chamber. Following washing steps, ligation and signal amplification was performed using Duo-Link II Detection Reagent Orange according to the manufacturer's instructions (Olink Bioscience). Slides were mounted in vectorshield/DAPI Mounting Media and observed using Zeiss LSM 510 confocal microscope. Results are shown like bright red dots and quantified using ImageJ software.

Statistical analyses

Statistical analyses were performed using Graphpad Prism 4.0 software (Graphpad software Inc.). Data are present as mean ± SD. Differences in the mean of samples were analyzed with the student´s *t* test. Differences under 0.05 were considered significant and indicated with a *.

Results

In this work we have investigated the role of the MUC1/S2 isoform in a pancreatic cancer model generated as briefly described in Material and Methods. **Figure 1** shows the expression levels of MUC1 in THPNE Mock and THPNE/S2 both by immunofluorescence **(A)** and Western blotting **(B)**. MUC1 levels are undetectable in the MOCK cell and in the MUC1/S2 clone it is possible to observe expression in 100% of the cells, mainly in the cytoplasm.

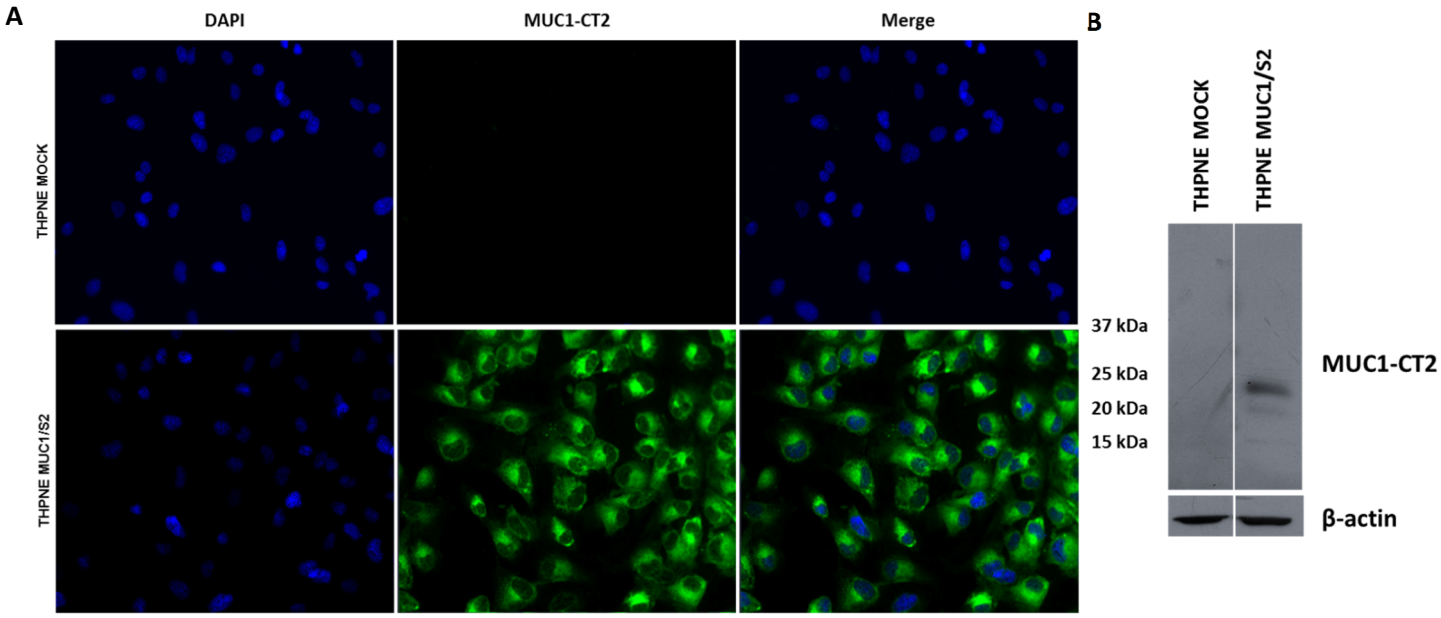

Figure 1. MUC1 expression in THPNE MOCK and THPNE MUC1/S2 transduced cells. Confirmation of MUC1 splice variant overexpression was performed by immunofluorescence **(A)** and Western blotting **(B)**, both using a MUC1-CT2 antibody against MUC1 cytoplasmic tail. For western blotting **(B)** β-actin was used as a control for protein loading.

We further studied the subcellular localization of MUC1/S2 isoform, using immunofluorescence co-localization study (**Figure 2**). We performed double immunofluorescence using an anti-MUC1 antibody and specific antibodies for mitochondria (anti-COXIV), Golgi apparatus (anti-Golgi Complex), endoplasmic reticulum (anti-Calveolin) and lysosomes (anti-LAMP1) and estimated the percentage of co-localization. The results obtained indicate that MUC1/S2 is present in all subcellular localizations to different degrees. More specifically, around 10% of MUC1/S2 expressed protein localizes in the mitochondria, 5% in the golgi apparatus, 25% in the endoplasmic reticulum and 10% in the lysosomes.

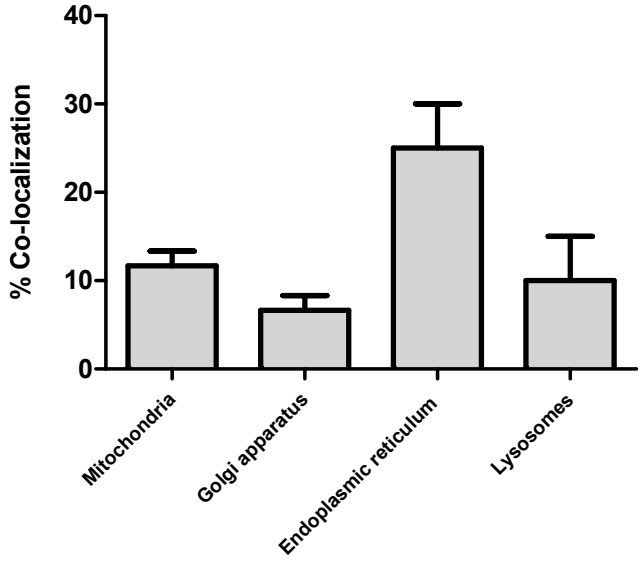

Figure 2. MUC1 localization within different cellular organelles by immunofluorescence confocal microscopy. The results represent the percentage of MUC1/S2 that co-localizes with specific proteins of each cell organelle.

We next analyzed the impact of MUC1/S2 splice variant *de novo* expression in this pancreatic cancer model using proliferation, migration and invasion assays. No differences were found in proliferation, determined by the BrdU assay, or migration, determined with the wound healing assay, when comparing MUC1/S2 transduced cells with the control **(Figure 3)**.

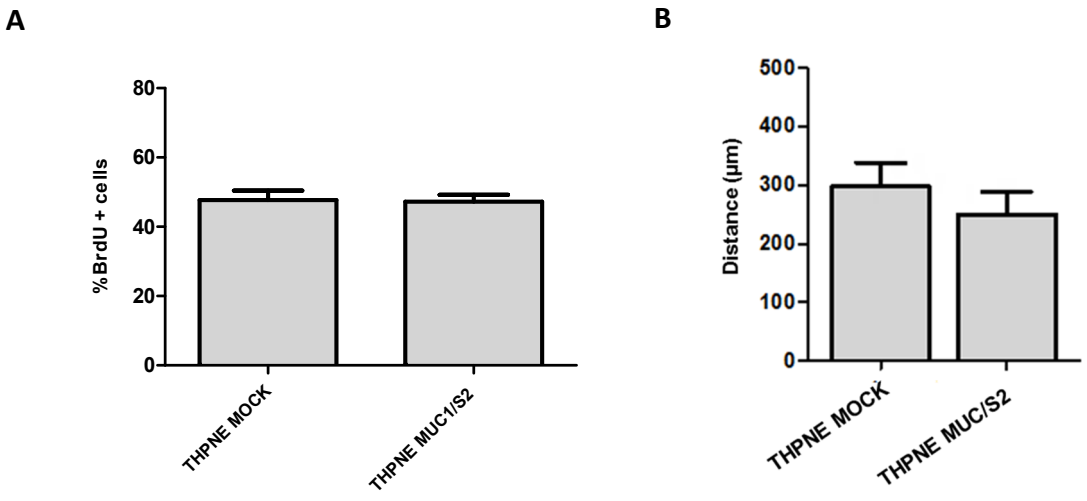

Figure 3. Cell proliferation and migration assay. (A) Cell proliferation analysis by the 5-Bromo-2'-deoxyuridine (BrdU) incorporation assay. Results are expressed as mean ± SD from three independent experiments. **(B) Cell migration analysis by wound healing *In vitro* assay.** Results are expressed as mean ± SD from three independent experiments evaluated 9h after wound.

To explore whether MUC1/S2 splice variant plays a role in the invasion capacity of the cells we performed a matrigel invasion assay **(Figure 4)**. The results show that the presence of the MUC1/S2 splice variant significantly increases the invasion capacity of THPNE cells (P<0.05).

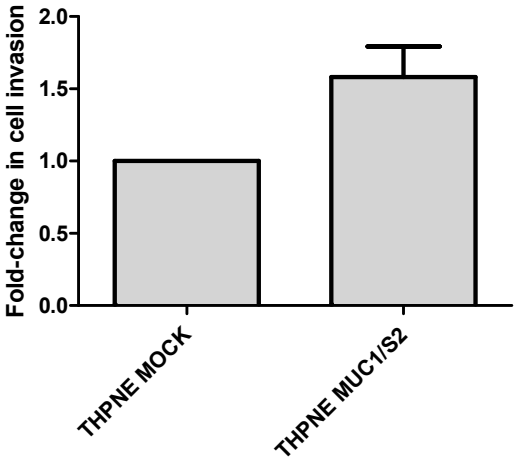

Figure 4. Matrigel invasion assay. Results are expressed as mean ± SD from three independent experiments. *, P<0.05; (Student's t-test).

Finally, we evaluated if the MUC1/S2 isoform retains the capacity to interact with p53 protein, which is known to be involved in MUC1 signaling **(Figure 5)**. Using PLA technique, we observed an interaction between MUC1/S2 and p53, mostly in the cytoplasm.

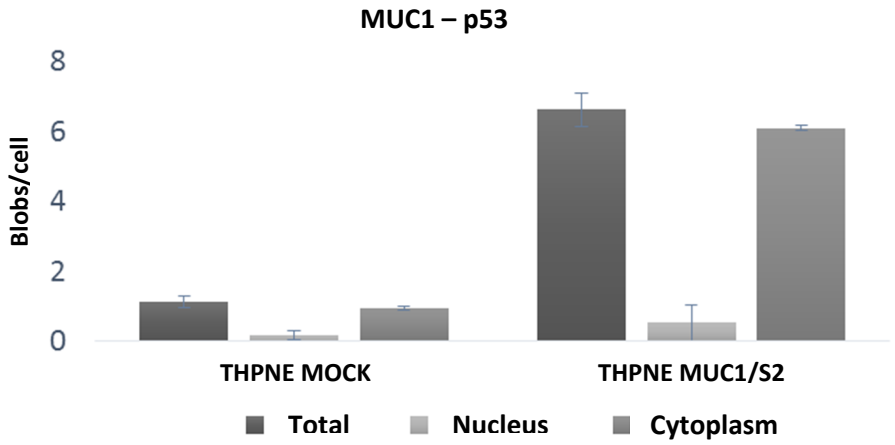

Figure 5. MUC1 – p53 interaction evaluation by proximity ligation assay (PLA). Results are expressed as mean ± SD from three independent slides.

Discussion

Pancreatic cancer remains a dismal disease for the vast majority of the patients representing a huge challenge both in research and in the clinical field, worldwide. Despite the efforts, conventional therapeutic approaches that include surgery, radiation, chemotherapy and even combinations of these have shown little impact on the progression of the disease. Facing this, the understanding of pancreatic molecular biology to address a better diagnosis, prevention and treatment is crucial. In this study, we aimed to determine the involvement of the MUC1/S2 splice variant in pancreatic carcinogenesis. To achieve this goal an *in vitro* model based on a transformed pancreatic cell line was used, transduced using a lentivirus system, with MUC1/S2 splice variant and MOCK construct.

We started by analyzing the expression level of MUC1 in the transduced cells. As a specific antibody for MUC1/S2 is not available, we used a primary antibody against MUC1 cytoplasmic tail (MUC-1 Ab-5 Mab) that is also present in MUC1/S2 isoform. It is possible to observe that 100% of the THPNE MUC1/S2 cells are expressing the transduced isoform and the expression is mostly restricted to the cytoplasm where it can be associated with other proteins playing a role in cell signaling (24). It is also possible to observe MUC1/S2 expression in the nuclei of the cells, but at much lower levels. MUC1/S2 localization in the nuclei indicates that although not having the cleavage region, MUC1/S2 is capable of translocation to the nuclei which points towards their participation in the modulation of transcriptional events that are involved in malignant progression, namely invasion and metastasis formation in response to, for example, Wnt/β-catenin or STAT pathways activation (13, 35). MUC1/S2 isoform is not present at the cell membrane, the normal cell localization of MUC1, which is expected, as this MUC1 isoform doesn't encompass the transmembrane domain. Therefore, biological processes related with membrane MUC1 localization such as interaction with EGFR, Erb2 and others RTKs contributing to activation of different pathways such as mitogen-activated protein kinase (MAPK) pathway in response to external environment are not expected to be activated by this MUC1 splice variant (18). MUC1 basal levels in THPNE MOCK cell line are undetectable, which validates our model for studying the impact of MUC1 splice variants.

To understand MUC1/S2 isoform distribution on the cell, which can give us an indication of its biological function, we performed a co-localization study. We evaluated its possible localization in the mitochondria, golgi apparatus, endoplasmic reticulum and lysosomes. We identified around 50% overall expressed protein in the cell compartments studied. Of these, the majority (around 25%) localizes in the ER. Endoplasmic reticulum has a central role in protein biosynthesis, determining the correct folding of a protein which affects its function and cellular localization and even if the protein will be secreted. Post-translational modifications, such as *N*-glycosylation, are processes that take place in ER and can be occurring to

MUC1/S2, explaining the observed 25% localization of MUC1/S2 protein in the ER. Due to the relevance of mitochondria in apoptosis, and the relevance of this process in carcinogenesis, it is important to further study MUC1/S2 presence in this subcellular compartment (around 10%). The additional 50% expressed protein are probably in the nuclei of the cell and in another compartments of cell cytoplasm like exosomes. Further studies are necessary to better understand the subcellular localization of this protein.

After evaluating the expression pattern and subcellular localization, we analyzed the impact of MUC1/S2 isoform in proliferation and migration, as they are relevant features associated to tumor cells in which full MUC1 is known to be involved. No differences were observed between THPNE MUC1/S2 and THPNE MOCK cell lines. Afterwards, we performed an invasion assay to analyze the possible involvement of the studied protein. MUC1/S2 overexpression led to an increased invasion of THPNE pancreatic cancer cells. The involvement of full MUC1 in invasion is documented and one of the described mechanisms show that once translocated to the nucleus MUC1 is capable of repressing E-cadherin expression and induce expression of EMT inducers such as Snail and Slug (23). Finally, we tried to understand if MUC1/S2 isoform retains the capacity to interact with p53 protein, which is known to be involved in MUC1 signaling. p53 is a major tumor suppressor gene involved in DNA repair, apoptosis, cell cycle arrest among others and is mutated in over 50% of human cancers (36). We observed an interaction between MUC1/S2 and p53, mostly in the cytoplasm. This interaction was described, in the nucleus, as contributing to the regulation of p53-responsive genes, namely, activation of *p21* and repression of *Bax* transcription, two proteins involved in cell-cycle progression and apoptosis (37). There are no studies showing the relevance of the interaction between MUC1 and p53 in the cytoplasm.

In conclusion, we validated the use of an *in vitro* model based on a transformed pancreatic cell line transduced with a MUC1 splice variant to study its relevance in pancreatic carcinogenesis. We evaluated the expression pattern of MUC1/S2 splice variant and its impact in proliferation, migration and invasion of the generated cells. Moreover, we assessed the subcellular localization of MUC1/S2 and its interaction with p53 protein. We observed that MUC1/S2 expression is mostly restricted to the cytoplasm and leads to an increase in the invasive phenotype of these cells. Excitingly, we identified for the first time an interaction between MUC1/S2 isoform and p53 protein, which function remains to be uncovered.

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Chapter 5

Reflections on MUC1 glycoprotein: the hidden potential of isoforms in carcinogenesis

Reflections on MUC1 glycoprotein: the hidden potential of isoforms in carcinogenesis

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Keywords: MUC1; cancer; cell differentiation; MUC1 splice variants

Abstract

Mucin 1 (MUC1) has been described as the renaissance molecule due to the large set of functions it displays in both normal and neoplastic cells. This membrane-tethered glycoprotein is overexpressed and aberrantly glycosylated in most epithelial cancers, being involved in several processes related with malignant phenotype acquisition. With a highly polymorphic structure, both in the polypeptide and glycan counterparts, MUC1 variability has been associated with susceptibility to several diseases, including cancer. Biochemical features and biological functions have been characterized upon the full-length MUC1 protein, remaining to clarify the real impact on cell dynamics of the plethora of MUC1 isoforms. This review aims to encompass a detailed characterization of MUC1 role in carcinogenesis, highlighting recent findings in cell differentiation and uncovering new evidences of MUC1 isoforms involvement in malignant phenotype.

Mucins

Mucins (MUC) are large and heavily O-glycosylated proteins generally expressed in the apical surface of epithelial cells being the major compound of the mucus. They are present in relatively harsh environments like the digestive and respiratory tracts and in the secretory epithelia of different organs such as the kidney, liver and pancreas (1). Mucin canonical functions are to provide protection from pathogens, dehydration, changes in pH and degradative enzymes. All mucins contain proline, threonine and serine (PTS)-rich tandem repeat domains that are heavily O-glycosylated in serine and threonine residues and contribute up to 50–80% of the glycoprotein total molecular weight (2). The specific sequence and the number of tandem repeats are variable among different mucins and individuals, due to genetic polymorphisms. N-glycosylation is also often present, although to a much lesser degree. The human MUC gene family encodes for up to 22 known proteins that can be divided in two main groups: secreted and membrane-associated mucins. Secreted mucins are subdivided in the gel-forming mucins (MUC2, MUC5AC, MUC5B, MUC6 and MUC19) and non-gel-forming mucins (MUC7) (3–7). Gel-forming mucins are typically expressed in specialized glands or goblet cells and their main function is to create the three-dimensional network of mucus, contributing for its viscoelastic properties through oligomerization domains, in order to protect the epithelium against different injuries (8). On the other hand, non-gel-forming mucins are smaller and unable to oligomerize, being mostly secreted by salivary and lachrymal glands (3, 9).

Membrane-associated mucins (MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20, MUC21 and MUC22), in addition to their function in protection of the epithelia from adverse conditions, also play important biological roles in cell-cell and cell-extracellular matrix interaction and signal transduction (10, 11). They are anchored at the cell membrane through a single hydrophobic transmembrane (TM) domain and, with the exception of MUC4, all of them contain a SEA (Sea urchin sperm protein, Enterokinase and Agrin) domain in the extracellular region (10). The expression profile of mucins as well as the glycosylation pattern is tissue- and cell type-specific and can be modified with cellular differentiation state or under pathologic conditions such as tumorigenesis.

Mucin 1

Structure and normal function

Mucin 1 (MUC1; also known as episialin, PEM, H23AG, EMA, MCA and CA15-3 among others) was the first mucin to be cloned and until now remains the best studied protein of this family. MUC1 is a membrane-associated mucin, normally expressed at basal levels in almost all epithelial tissues, being found in most human organs, namely, the esophagus, stomach, liver, pancreas, breast, lungs or kidney (12). Moreover, it was also described in hematopoietic and human embryonic stem cells (hESCs) as well as in stem/progenitor breast cells (13–16). MUC1 is normally expressed at the apical surface of polarized epithelial cells in relatively harsh environments like the digestive and respiratory tracts to provide protection from pathogens, dehydration, changes in pH and degradative enzymes, functions that are enhanced by its extensive glycosylation.

MUC1 gene is located on the long arm of chromosome 1 in position 21 (locus 1q21), and comprises seven exons ranging about 4.4 kb. The mature MUC1 protein encompasses two subunits, the extracellular N-terminal alpha subunit and the mainly intracellular C-terminal beta subunit. The N-terminal subunit contains the important signal peptide that localizes the mature protein to the apical membrane and the variable number tandem repeat (VNTR) domain. On the other hand, the C-terminal subunit is composed of an extracellular stem region of 58 amino acids (aa), a short transmembrane domain with 28 aa and the cytoplasmic domain (MUC1-CD) with 72 aa (Fig. 1) (17, 18). The VNTR region consists of a serine and threonine-rich highly polymorphic region composed by closely identical sequences of 20 amino acid (aa) repeated 25 to 125 times, being the most common the PAHGVTSAPDTRPAPGSTAP sequence (17). The variable number of VNTRs and their content of Ser/Thr residues can be markedly different among individuals and is associated with different susceptibility to several diseases, including cancer and precursor lesions (19–21).

MUC1 is synthesized as a single polypeptide chain being autoproteolytically cleaved in two subunits (alpha (α) and beta (β)) in the endoplasmic reticulum soon after synthesis and is present, in normal conditions, on the cell surface as a heterodimer (22, 23). During MUC1 folding, the cleavage of the precursor polypeptide occurs between glycine and serine residues of the GSVVV motif within the SEA module of the extracellular domain (22, 24, 25). MUC1 core protein has a molecular weight of 120–225 kDa which changes to 250–1000 kDa with glycosylation and, in the membrane, the subunits α and β bind together through a strong non-covalent interaction (26, 27). Identical to other mucins, MUC1 is mostly O-glycosylated showing fewer N-linked glycans. Serine and threonine residues are the targets of O-linked glycosylation and in the VNTR region each sequence contains up to five glycosylation sites. The glycosylation profile of MUC1 is dependent on the tissue and on the profile of glycosyltransferases expressed (28, 29). MUC1, like other membrane-associated mucins, besides playing a central role in maintaining homeostasis and promoting cell survival in response to harsh environments, is also involved in cell signaling transduction events mostly through MUC1-CD phosphorylation. MUC1-CD, a highly conserved 72 aa sequence, contains several Tyr residues that represent potential docking sites for proteins with SH2 domains, such as protein kinase C δ (PKCδ), glycogen synthase kinase 3β (GSK-3β), ErbB receptors like ErbB1/epidermal growth factor receptor (EGFR) and some family members of c-Src non-receptor tyrosine kinases Lyn and Lck (30–32). ZAP-70 tyrosine kinases are also responsible for MUC1-CD phosphorylation in response to T-cell activation (33). The serine, threonine and tyrosine residues of MUC1-CD represent potential phosphorylation sites and changes in the phosphorylation status of MUC1-CD can modulate its affinity for mediators of signal transduction, including β-catenin and p53 proteins (**Fig. 1**) (34, 35).

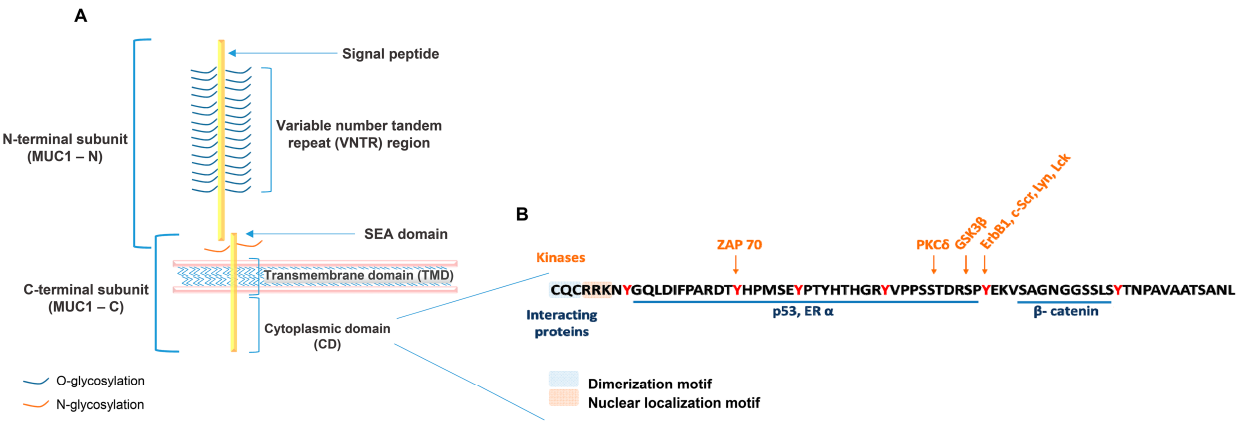

Fig. 1. MUC1 structure with sequence of cytoplasmic domain and putative binding sites. (A) N-terminal subunit and C-terminal subunit forming a stable heterodimeric complex at the cell membrane. **(B)** Amino acid sequence of MUC1 cytoplasmic domain (MUC1-CD), showing potential phosphorylation sites and protein-binding motifs.

MUC1 alterations and function in cancer

MUC1 is one of the most deregulated mucins during carcinogenesis being overexpressed in most human epithelial cancers and hematologic malignancies. In tumor cells, MUC1 displays new biochemical features, cellular distribution and functions.

MUC1 is overexpressed in tumors due to several mechanisms including increased transcription, amplification of MUC1 genomic locus or loss of post-transcriptional regulation (36–39). In tumor cells, MUC1 has fewer, shorter and less branched glycans which is in contrast with normal cells that normally show more elongated and highly branched glycans (40). The hypoglycosylation of MUC1 in cancer cells impacts on the stability and the subcellular localization of the protein increasing intracellular uptake by clathrin-mediated endocytosis. As a consequence, and since MUC1 degradation is not increased, there is an intracellular accumulation of this protein (41). MUC1 glycosylation has also been associated with inflammatory response since its dense glycan component entraps proinflammatory factors such as transforming growth factor α (TGF-α), interleukin 1 (IL-1) and IL-4, among others, that are released after MUC1-N shedding, inducing an inflammatory response (42). Due to the loss of polarity in tumor cells, MUC1 is no longer restricted to the apical surface and covers the entire cell surface. This loss of polarity disturbs cell-cell and/or cell-matrix interactions favoring the release of tumor cells into the blood circulation and enabling the interaction between MUC1 and other membrane proteins normally expressed in the basolateral domain of the cells such as growth factor receptors (43). Interactions with these proteins can contribute to growth and survival of tumor cells through activation of signaling pathways or by blocking the access of other molecules to these receptors (**Fig. 2**) (44).

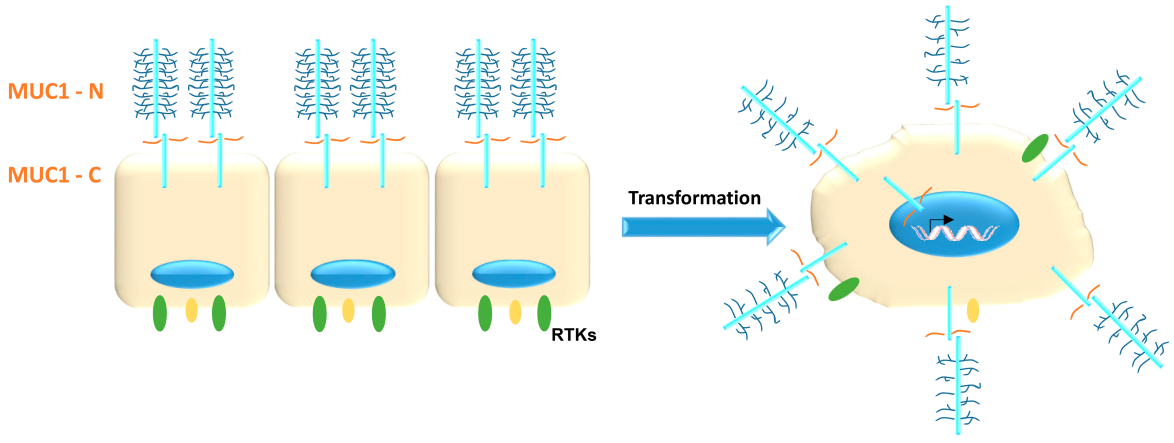

Fig. 2. MUC1 properties and subcellular localization. MUC1 is confined to the apical surface of normal epithelial cells. However, in tumor cells, this polarity is lost and no longer restricted to the apical surface covering instead the entire cell surface, which potentiates the interaction between MUC1 and receptor tyrosine kinases (RTKs). These interactions could contribute to signaling pathway activation or repression by blocking the access of other molecules to these receptors. Hypoglycosylation of MUC1 is also a feature in cancer cells.

Although the non-consensual recognition of MUC1 as an oncogene, its involvement in proliferation, angiogenesis, invasion and metastization is now well documented. Studies in transgenic mice and in different cell lines have related MUC1 with carcinogenesis in multiple ways. It has been hypothesized that, globally, MUC1 participates in the control of local microenvironment allowing the tumor to adapt, survive and proliferate in challenging contexts during invasion and metastasis. MUC1 does not have a DNA binding domain but interacts directly with a diversity of transcription factors making part of transcriptional complexes and promoting their specificity. This regulation is significantly modulated by MUC1-CD modifications (23, 45, 46). The discovery that overexpression of MUC1-CD alone is sufficient to induce anchorage independent growth and tumorigenicity of carcinoma cells in vitro led to a stronger focus on the study of this MUC1 subunit (47). MUC1-CD signaling at the cell membrane is triggered by phosphorylation of the serine and threonine residues. This phosphorylation can occur in response to the activation of several surface growth factor receptors, namely, platelet-derived growth factor receptor (PDGFR) and members of the Erb family which include EGFR and ErbB2/Her2 (43, 45, 48). MUC1-CD works as a docking site for the activation of different signaling pathways important in cellular proliferation, differentiation, migration and survival such as PI3K and mitogen-activated protein kinase (MAPK) pathways (49). MUC1-CD is also a target of several different kinases including the PKCδ, GSK3β and the tyrosine kinases c-Src and Lck (50). Phosphorylation events regulate the interaction between MUC1-CD and other intracellular binding partners such as effectors of the Wnt pathway or β-catenin (30, 51, 52). MUC1 and β-catenin do not interact in a normal polarized epithelium since they have different subcellular localizations. β-catenin binds to MUC1-CD through a motif similar to those present in E-cadherin and in the adenomatous polyposis coli (APC) protein, at the SSSL sequence (52). This interaction is dependent of MUC1-CD phosphorylation by c-src kinase and is inhibited by GSK3β phosphorylation (30). MUC1-CD/β-catenin complex leads to an accumulation of β-catenin in the cell nuclei where it acts as a transcriptional coactivator of genes controlling cellular growth such as cyclin D1 and c-myc, promoting tumor progression (53). Importin β and nucleoporin Nup62 are the mediators of MUC1-CD nuclear translocation (54). Moreover, MUC1-CD/ β-catenin interaction decreases the ability of β-catenin to interact with E-cadherin at adherens junctions leading to a decrease in cell-cell interaction (55). β-catenin in the nucleus also represses E-cadherin expression and increases the transcription of epithelial-mesenchymal transition (EMT) inducers such as Snail, Slug, Vimentin and Twist, enhancing the invasive potential of tumor cells (56, 57). EMT is also induced by MUC1 through regulation of miRNAs involved in EMT-gene expression (58, 59).

Angiogenesis is another hallmark of cancer in which MUC1 plays a role. The growth of the tumor mass creates a low oxygen and nutrient microenvironment (hypoxia) that induces tumor cells to express proangiogenic factors, promoting growth of new vessels in order to adapt and

survive in this environment. It has been demonstrated that MUC1 stimulates the expression of proangiogenic factors like connective tissue growth factor (CTGF), platelet-derived growth factor subunit B (PDGF-B) and vascular endothelial growth factor A (VEGF-A), that are responsible for the synthesis of new blood vessels within the tumor (60). In pancreatic cancer, it was also reported that MUC1 overexpression induces the synthesis and secretion of VEGF through the AKT signaling pathway (61). Due to the loss of apical-basal expression restriction, in cancer MUC1 regulates the cell metabolite flux at several levels. These metabolic alterations provide tumorigenic features to the tumor cells such as proliferation and metastasis formation (62). MUC1 is involved in glycolysis regulation through the PI3K/Akt pathway that induces expression of glucose transporters and stimulates the activity of hexokinase and phosphofructokinase. As a result, MUC1 increases glucose uptake and lactate production stimulating glycolysis (63). It was also demonstrated that MUC1 occupies multiple promoters of genes directly involved in glucose metabolism and is responsible for an increased expression of glycolytic genes both in normoxia and hypoxia conditions in a hypoxia-inducible factor-1 alpha (HIF-1α) dependent and independent manner (64). MUC1 overexpression also confers cells survival advantage by blocking cell death mediated by both the intrinsic and extrinsic apoptotic pathways. MUC1-CD is able to bind to heat shock proteins 70 or 90 (HSP70 or HSP90). They are translocated as a complex to the mitochondria where MUC1-CD blocks the loss of mitochondrial transmembrane potential abolishing apoptotic response to oxidative, genotoxic, hypoxic and metabolic stress (65–67). Direct interaction between p53 and MUC1-CD was also reported in response to genotoxic stress, promoting the transcription of p53-target genes (68). A recent study showed that MUC1 overexpression in pancreatic cancer cells increases chemoresistance by upregulating multidrug resistance genes, particularly, the ABCC1 gene that encodes for the multidrug resistance protein 1 (MRP1) (69). The involvement of MUC1 in tumor cell proliferation, migration, invasion, cell survival and chemoresistance and its overexpression in a large number of cancers, namely, gastric, pancreatic and breast, make it an elective candidate for targeted therapies and big efforts are being made in this field. Presently, MUC1 is used as a biomarker for cancer staging and follow-up, as it is released from tumor cells into the circulating serum of cancer patients after cleavage of the extracellular domain.

MUC1 and cell differentiation

MUC1 is normally expressed at basal levels in almost all epithelial tissues and is also expressed in non-epithelial cells including B and T hematopoietic cells (13, 15). It is known that hematopoietic stem cells are enriched in MUC1 expression and its overexpression in cord blood cells is sufficient to increase both progenitor and long-term culture initiating cell rates (70). Reactive oxygen species (ROS) are critical in the regulation of self-renewal in normal

hematopoietic cells and MUC1 is known to regulate intracellular levels of ROS (71–74). MUC1 is also aberrantly expressed in blasts from patients with acute myelogenous leukemia (AML), which is associated with unlimited self-renewal and blockage of terminal differentiation (70, 75). Yin and colleagues described that the inhibition of MUC1-CD with the GO-203 inhibitor, a cell-penetrating peptide that binds to the CQC motif, induces the terminally differentiated myeloid phenotype in AML cell lines and primary blasts (76). This study further shows that the inhibition of MUC1-CD increases hydrogen peroxide and superoxide levels and depletes glutathione, that are known to induce terminal cell differentiation. These findings suggest the involvement of MUC1 in the regulation of the redox balance in AML cells while other studies suggested MUC1 as a good candidate target in the treatment of AML (77). In chronic myeloid leukemia (CML), triggered by the expression of the Bcr-Abl fusion protein (78), MUC1 is expressed only during the blast phase, the more aggressive stage of this disease (79). In this disease model, a different MUC1-CD oligomerization inhibitor, GO-201 inhibitor, is associated with downregulation of Bcr-Abl levels and induction of terminal cell differentiation in CML (80).

Hikita et al. showed that undifferentiated human embryonic stem cells (hESCs) do not express the full length MUC1 protein but rather a low molecular weight cleaved product, MUC1* (14). MUC1* retains only 45 aa of the extracellular domain, harbors a growth factor receptor-like activity and is the predominant form of the protein expressed in breast tumor tissues and cell lines (81). The NM23 ligand of MUC1* in cancer cells co-localizes with pluripotent cells while newly differentiated stem cells express the full-length MUC1. In pluripotent stem cells, MUC1* functions as a growth factor receptor in a βFGF independent manner and may be a modulator of cell differentiation (14). More recently, the role of MUC1* in induced pluripotent stem (iPS) cells was also studied. Both in iPS and hES cells, NM23-H1 dimer binds to and dimerizes MUC1*, promoting growth and pluripotency. On the other hand, NM23-H1 hexamer is not capable of MUC1* dimerization inducing differentiation instead, which makes this complex capable of acting both as a differentiation repressor or inducer (82).

It is currently accepted that a small subpopulation of cells designated cancer stem cells (CSCs) or tumor-initiating cells have self-renewal capacity and the ability to give rise to the heterogeneous cellular subpopulations identified in tumors. This subpopulation of cells is believed to have the exclusive ability to drive tumor formation, growth, relapse and chemoresistance, being also identified in an ever increasing number of human tumors (83–86). Therefore, CSCs have been described as potentially elective targets for therapeutic strategies (87). More recently MUC1 has been associated with biological properties attributed to these cells, although the correlation between MUC1 and CSCs phenotype remains to be fully elucidated.

MUC1 was reported to be expressed in pancreatic and acute myeloid leukemia CSCs and to keep hESCs in an undifferentiated stage (14, 77, 88). MUC1 is upregulated by Extracellular Matrix Protein 1 (ECM1) which in turn increases the CSCs phenotype of breast tumor cells through stabilization of b-catenin protein (89). In lung cancer, using in vitro and in vivo models, Ham et al. reported that MUC1-CD is overexpressed in paclitaxel (PTX) resistant tumor cells. These authors showed that overexpression of MUC1 was involved in stemness features such as self-renewal, proliferation and sphere-forming ability (90). Moreover, it was reported that MUC1 contributes to the self-renewal capacity of breast cancer cells by acting in the NFKβ pathway and that the inhibition of MUC1 with MUC1-CD GO-203 inhibitor decreased this capacity (91). Also, in a breast cancer model, it was demonstrated that an isolated “side population” was highly enriched in stem/progenitor cells that show overexpression and hypoglycosylation of MUC1 (92).

MUC1 isoforms and their role in cancer

Most efforts in MUC1 research have been focused on the full MUC1 protein and its role in cancer initiation and progression. However, MUC1 undergoes alternative splicing events that give rise to different isoforms with distinct biological roles (**Fig. 3**).

Alternative splicing is a key process in the regulation of gene expression contributing to the heterogeneity of the transcriptome and the proteome and is estimated to occur in over 95% of all multiple-exon human genes. This mechanism generates multiple mRNAs from a single gene during pre-mRNA maturation and results in a variety of proteins that differ in their amino acid sequence. Alternative splicing has been associated with a large number of diseases, including cancer (93). The best characterized MUC1 isoform is the polymorphic mucin-like type 1 transmembrane protein, MUC1/REP or MUC1/TM, which encodes the full molecule. However, MUC1 has different isoforms generated by alternative splicing pathways including exon skipping, full or partial intron retention, alternative 50 splice sites and alternative 30 splice sites (94). Although more than 70 isoforms have been identified so far, the full-length sequence has only been reported for some.

MUC1/SEC, a secreted truncated isoform, retains the VNTR region and is devoid of the transmembrane and cytoplasmic regions. This isoform is secreted by different cell types, like breast and colon cells, benign ovarian tumors and is also present in the serum of breast cancer patients and in human milk (95–98).

Recently, 78 different MUC1 isoforms were isolated from a panel of human cervical (HeLa) cells, breast cancer (MCF7) cells, T-cell leukemia Jurkat cells and from human activated T cells, being MUC1/A, MUC1/B, MUC1/C, MUC1/D, MUC1/X, MUC1/Y and MUC1/ZD the more abundant ones (94). This study identified exon 2, which contains the VNTR region, as the most skipped exon among all the different isoforms using the same 50 splice site and variable 30

splice sites. The authors point several different reasons for this to occur, namely, the large size of this exon which can be as high as 6200 bp; the presence of core splicing signals such as the 50 splice site, the 30 splice site and the branch point site in the VNTR sequence; the presence of cis-regulatory elements at the junction of the repeats that have been described as intron splice; the existence of Nova splicing factor binding sites (YCAY, with Y being any pyrimidine) in each repeat, which can result in a blockage of exon inclusion when bound by Nova. On the other hand, MUC1-CD region remains unchanged in almost all MUC1 short isoforms, allowing them to keep the important signaling function (94).

MUC1/A, MUC1/B, MUC1/C and MUC1/D encode a “full-length” MUC1 having a fixed splice donor site near the 50 end of intron 1 and variable splice acceptor sites near the 30 and 50 ends of intron I and exon 2, respectively. MUC1/A retains 27 bp of intron I without causing a reading frameshift while MUC1/B has intron I completely excised. MUC1/C and MUC1/D show excision of portions of exon 2, 9 bp and 35 bp respectively, keeping the reading frame (99, 100). MUC1/A was initially described in thyroid carcinoma tissues, is usually associated with malignancy and is expressed preferentially in ovarian cancer when compared to benign tumors (97, 101). On the other hand, MUC1/B was described as having significantly higher expression in normal tissue and is associated with better prognosis in tumors of the esophagus (102). However, there are few reports describing the expression pro-file of MUC1/A and MUC1/B in tumors which hampers an accurate evaluation of the relevance of these two splice variants in carcinogenesis.

MUC1/Y, MUC1/X and MUC1/ZD splice variants arise from a fixed splice donor at the 50 end of exon 2 and different splice acceptor sites around the 30 end of exon 2 resulting in MUC1 isoforms that lack the VNTR region but retain the transmembrane and cytoplasmic domains. MUC1/Y, an uncleaved splice variant, does not exhibit mucin-like features. However, it is expressed by diverse human secretory epithelial tumors, being undetectable in the adjacent normal tissue. It was also shown to be involved in tumor initiation and progression, in vivo, in breast cancer (103–106). It was described that MUC1/Y undergoes tyrosine/serine phosphorylation being potentially able to interact with proteins containing a SH2 domain, such as Grb2, triggering a cell signaling cascade and functioning in a similar way to cytokine receptors (104, 107). MUC1/SEC is capable of binding to MUC1/Y in a region homologous to the ligand binding sites of cytokine receptors, inducing its phosphorylation and stimulating cell signaling pathways (104). MUC1/Y was also shown to be overexpressed in ovarian and prostate cancer, being also associated with breast cancer (108–110).

MUC1/X and MUC1/ZD were also associated with malignant ovarian tumors (97). MUC1/X, like MUC1/Y, misses the VNTR region but has a different acceptor site 18aa upstream to MUC1/Y. It is highly expressed in cervical and ovarian cancer cells and contrary to MUC1/Y this isoform undergoes proteolytic cleavage (106, 108, 111, 112). MUC1/ZD also

lacks the VNTR region but is comprised of a unique C-terminal sequence of 43 aa resulting from a shift in the reading frame (106).

More recently, a truncated genomic fragment of human MUC1 was shown to induce epithelial to mesenchymal transition in mammary mouse cells (113). In addition, a MUC1 transmembrane cleaved form (MUC1*) was reported to have an important role in chemoresistance to standard chemotherapy agents (114) as well as to be an accurate marker of pluripotency in human embryonic stem cells while full-length MUC1 is present in newly differentiated stem cells as mentioned before (14). This isoform contains only 45 aa of the MUC1 extracellular domain, lacking the VNTR region.

MUC1/S2 isoform was identified in human cervical (HeLa) and breast cancer (MCF7) cell lines and is absent in T-cell leukemia Jurkat cells and human activated T cells (94). This splice variant has a retention of 27 bp of intron I, excision of 659 bp from exon 2 and total excision of exons 3, 4 and 5 lacking the transmembrane domain and cleavage site of the SEA domain. We have investigated the role of MUC1/S2 isoform in a pancreatic cancer model. For that, we used a transformed hTERT-THPNE (THPNE) cell line derived from a non-tumorigenic epithelial pancreatic duct and immortalized with the catalytic subunit of telomerase (115). THPNE cell line was generated, as previously described, by sequential retroviral mutant K-Ras (G12D), E6/E7, and SV40 small t antigen transduction in order to mimic some of the oncogenic alterations present in the current accepted pancreatic cancer progression model (115, 116). Transfection of MUC1/S2 sequence (AY327584) into the THPNE cell line allowed the observation that MUC1/S2 expression is mostly restricted to the cytoplasm and to a lesser extent to cells nuclei. MUC1/S2 led to increased invasion of these pancreatic cancer cells and interestingly, it still interacted with p53 despite not having the full protein structure, which can indicate a role of this isoform in signaling pathways (unpublished data). Further studies are necessary to disclose the complete role of this MUC1 splice variant in pancreatic cancer.

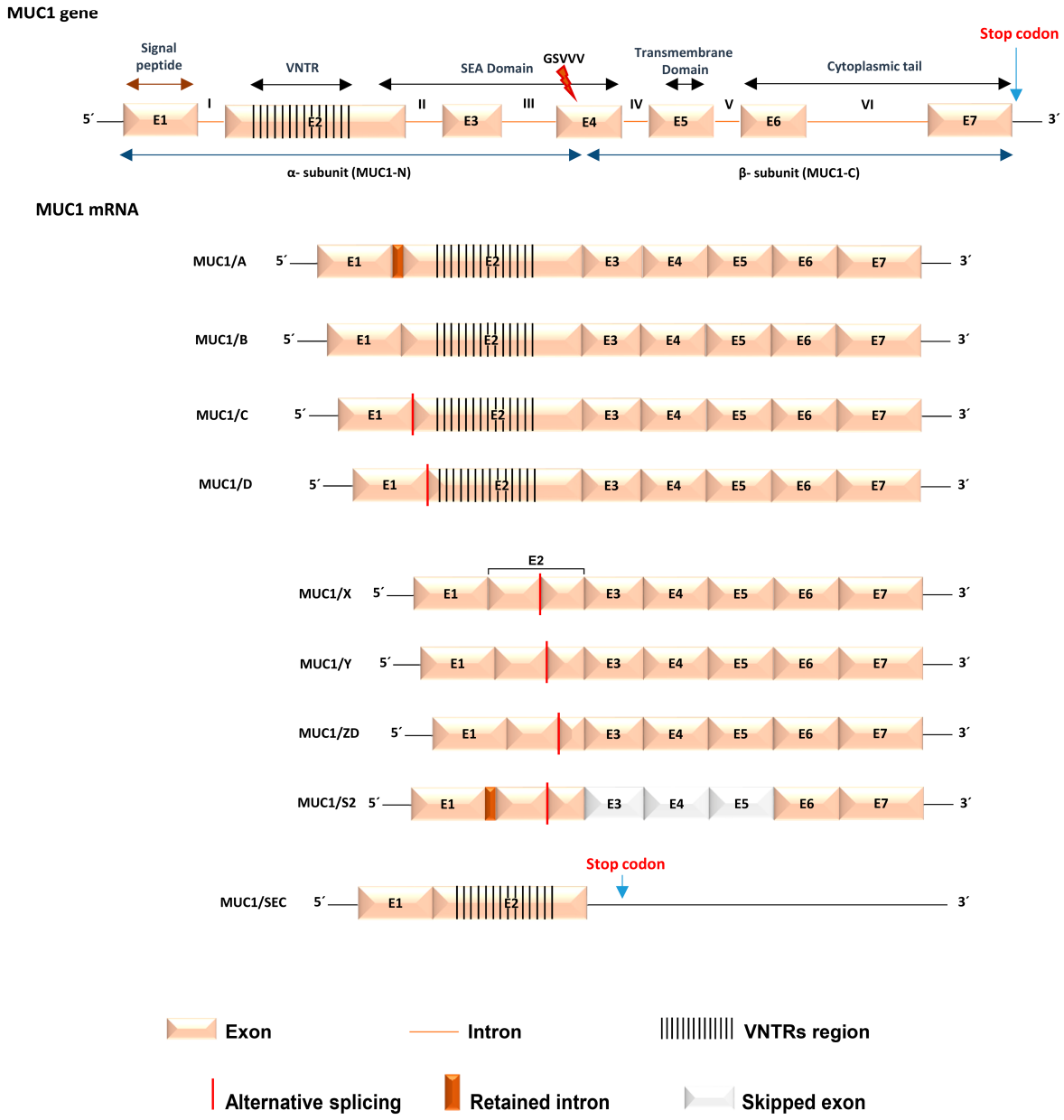

Fig. 3. Representation of MUC1 gene with respective protein domains and different mRNA splice variants. MUC1 gene comprises seven exons (E1-E7) and six introns (I-VI). The respective protein domains encoded by each exon region are represented above the exon. The mRNA of different splice variants is schematized. MUC1/A, MUC1/B, MUC1/C and MUC1/D represent the four main variants and have distinct 30 splice acceptor sites in exon 2. MUC1/X, MUC1/Y and MUC1/ZD splice variants completely lack the VNTR region. MUC1/S2, besides the VNTRs excision lacks exons 3, 4 and 5. MUC1/SEC splice variant, which retains the VNTR region and has a premature stop codon within intron II is also represented.

Conclusions and Future challenges

MUC1 has been associated with oncogenic events in many epithelial tumor models. The full extent of such involvement has been investigated in different biological contexts including tumor cell survival, proliferation, differentiation, migration and invasion. The newly identified MUC1 functions expose the ubiquitous role of this mucin in the carcinogenesis process, both in initiation as well as in progression and metastasis. The growing scenario of a multi-task MUC1 reflects the structural and functional complexity of the molecule, nonetheless the identification of MUC1 functions that drive the neoplastic transformation and those that derived from it, remains rather challenging.

The available evidence collected so far remains limited to answer questions about MUC1 overexpression and abnormal glycosylation in tumor cells, or the relevance of MUC1 in oncogenic signaling cascades and transcriptional regulatory networks. More recently, insights about MUC1-mediated chemoresistance, tumor cell metabolism, epithelial to mesenchymal transition (EMT) and stem cells phenotype further increase the current challenges that both basic and clinical researchers are facing. Addressing these questions will be essential to progress on the fully comprehension of this molecule impact on cancer phenotype and to expand the panel of new MUC1-based clinical approaches.

MUC1 is an elective target for cancer immunotherapy due to the high protein expression and abnormal forms associated with malignancy. A diverse assortment of MUC1-based therapeutic strategies have been investigated lately, encompassing monoclonal antibodies (MAb) and vaccines, aptamers and nanoparticles (117, 118). The Mab based therapies and vaccines have been previously focused on MUC1-N VNTR aberrant glycosylation, but lately a progressive shift occurred and the new generation of MAb and vaccines is targeted to MUC1-C. The cytoplasmic region of MUC1 is also the focus of aptamers designed to inhibit dimerization and phosphorylation motifs present in this region. Clinical trials are currently evaluating and optimizing these new therapies in different tumor models (119–121). Similarly, MUC1 has been found to be a useful biomarker for prognostic and follow-up, due to the presence of MUC1 in the membrane of circulating tumor cells or in the serum of cancer patients (122, 123).

Understanding the pattern of expression, subcellular localization, biological functions and diagnostic/prognostic value of the different MUC1 isoforms is an essential step to clarify MUC1 role in the different hallmarks of cancer. Despite a few reports that revealed the existence of a vast amount of different MUC1 splice variants, most of the previous studies addressing MUC1 isoforms were PCR technology based and thus very unspecific on discriminating MUC1 splice variants that share gene sequences. The evidences gathered so far reinforce the relevance and ubiquity of MUC1 in neoplastic phenomena; still it urges to develop new tools (e.g.,

antibodies) and mobilize the current available technologies (e.g., next generation sequencing) to disclose the full spectrum of MUC1 isoforms in malignant cells. Our group has been focused and will pursue the study of MUC1 variants relevance for the biology of normal and pancreatic tumor cells using engineered in vitro models that mimic the pancreatic carcinogenesis.

The polymorphic nature of MUC1 molecule, enhanced by the tumor-specific alterations, and the vast underlying transcriptome, encompasses a unique potential waiting to be translated into innovative therapies of personalized medicine.

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Chapter 6

General Discussion

Pancreatic cancer remains a dismal disease for the vast majority of the patients representing a huge challenge worldwide both in basic research and in the clinical field. Despite the efforts, conventional therapeutic approaches that include surgery, radiation, chemotherapy and even a combination of these three, have shown little impact on the development of the disease. Facing this, the understanding of pancreatic cancer molecular biology to address a better diagnosis, prevention and treatment is crucial. In this study, we aimed to determine the involvement of MUC1 in the CSC phenotype and to investigate biological functions of MUC1 splice variants in pancreatic carcinogenesis, two considerably underdeveloped fields in pancreatic carcinogenesis.

Alterations in glycans profile and in mucin expression levels are common amongst adenocarcinomas including pancreatic. MUC1, the best characterized mucin, is overexpressed in around 80% of all PC showing a different glycosylation profile and cell expression distribution. Despite MUC1 involvement in tumor progression, metastasis and poor prognosis being well characterized and extensively revised, its role in carcinogenic initiation is not fully known. A recent study reported that a transmembrane cleaved form of this protein (MUC1*) is expressed in pluripotent human embryonic stem cells (hESCs) working as a growth factor receptor. This leads to increased proliferation and prevents cells from differentiation upon binding to a metastasis associated protein, NM23-H1 (Hikita et al. 2008, Mahanta et al. 2008). More recently, Mukherjee P. and colleagues showed that a new monoclonal antibody against the tumor-associated MUC1 protein was able to identify CSCs in patients with PC (Curry et al. 2013).

The reported associations between MUC1 and CSCs led us to investigate the potential contribution of MUC1 to oncogenic signaling pathways in these cells. Several markers have been used to identify and isolate CSCs from different tumor types including colon, prostate, breast and pancreas (Al-Hajj et al. 2003, Collins et al. 2005, Dalerba et al. 2007, Hermann et al. 2007). In pancreas, the markers used for CSC isolation were the combination of $CD44^+$ $CD24^+$ ESA^+ cell surface markers and CD133 surface markers (Hermann et al. 2007, Li et al. 2007, Moriyama et al. 2010). Based on these markers, the subpopulations isolated showed a higher tumorigenic and self-renewal capacity as well as higher resistance to standard chemotherapy and a close relationship with the metastatic phenotype (Hermann et al. 2007, Moriyama et al. 2010).

Human CD133 is a 120kDa cholesterol-interacting pentaspan-transmembrane glycoprotein, expressed in plasma membrane protrusions, also known as Prominin-1. CD133 protein was initially described as a cell surface antigen specific of hematopoietic stem and progenitor cells as well as bone marrow-derived circulating endothelial progenitors involved in angiogenesis, inflammation and tissue regeneration (Asahara et al. 1997, Miraglia et al. 1997, Yin et al. 1997, Shaked et al. 2006). Although the involvement of CD133 in the maintenance

of stem cells properties has been previously reported, its biological function remains controversial and under debate (Marzesco et al. 2005, Sgambato et al. 2010). However, some reports showed a correlation of this marker with poor PC prognosis and other cancer types (Kojima et al. 2008, Maeda et al. 2008, Chen et al. 2013, Okamoto et al. 2013). In pancreas, it was described that spontaneous LSL-Kras$^{G12D/+}$; LSL-Trp53$^{R172H/+}$; Pdx-1-Cre (KPC) mouse model, an aggressive model with 100% penetrance and about 6 months survival, has approximately 7–8% of CD133^{+} cells that showed an increased expression of prosurvival and proinvasive proteins when compared with CD133^{-} cells (Hingorani et al. 2005, Banerjee et al. 2014). In CSCs, namely in pancreatic CSCs, it is know that several canonical signaling pathways involved in self-renewal, tumor growth, invasion, metastization and chemoresistance are altered and it is argued that the inefficiency of current therapies is due to the failure of eradicating this subpopulation of cells (Castellanos et al. 2013). Overexpression of CD133 in a pancreatic cancer cell line showed an increase in CSC phenotype and upregulation of several genes related with “stemness” such as *NANOG*, *MYC* and *SOX2,* among others (Nomura et al. 2015). Hence, as a first objective of this thesis we evaluated the involvement of MUC1 and its signaling partners in the phenotype of pancreatic CSCs in order to better understand the mechanisms that regulate the specific biological properties of this subpopulation of cells. With this purpose, we isolated pancreatic CSCs, using a well-established magnetic cell sorting (MACs) method, based on the CD133 surface marker assay and using a moderately differentiated PDAC cell line, HPAF-II. This cellular model, derived from the ascitic fluid of a patient with PDAC, offers the advantage of being a low-passage cell line that more accurately mimics the primary tumor (Metzgar et al. 1982). Our results demonstrated that the isolated CD133^{+} cell subpopulation show well defined features of CSCs (tumorigenicity, tumor phenotype recapitulation) and concomitantly have increased expression of MUC1. MUC1 is involved in cell signaling-transduction events mostly through MUC1-CD phosphorylation. MUC1-CD encompasses 72 amino acids that comprise several serine and tyrosine residues which can be extensively phosphorylated by different proteins with SH2 domains, such as PKCδ, GSK-3β and EGFR (Pandey et al. 1995, Quin and McGuckin 2000, Li et al. 2001). Changes in the phosphorylation status of MUC1-CD modulate its affinity to interact with mediators of signal transduction, triggering signaling pathways and leading to changes in gene expression and consequently, cell phenotype (Ren et al. 2002, Wei et al. 2007). One of these mediators is β-catenin, which binds directly to MUC1-CD SAGNGGSSLS motif in the armadillo repeats (Yamamoto et al. 1997, Huang et al. 2003).

In this work, we characterized the expression of EGFR, PKCδ, GSK-3β and GRB2 kinases that are known to be involved in MUC1 signaling pathways. In the CD133^{+} cells subpopulation, an increased expression of EGFR and PKCδ was observed while GSK-3β expression was decreased. It is known that MUC1-CD phosphorylation by EGFR and PKCδ promotes

interactions between β-catenin and MUC1, while phosphorylation by GSK3β leads to a decrease in this association (Li et al. 1998, Schroeder et al. 2001). Indeed, we were also able to identify an increased MUC1-CD/β-catenin interaction in the $CD133^+$ cell subpopulation despite unchanged levels of steady state β-catenin levels in these cells. This increased interaction is observed both in the $CD133^+$ cell subpopulation and derived xenograft. It is known that MUC1/β-catenin complex is involved in several tumorigenic processes being responsible for the activation of the Wnt/β-catenin signaling pathway. In epithelial cells, β-catenin is a crucial element of the adherens junction that associates cadherin adhesion molecules located at the cell surface to the actin cytoskeleton. MUC1-CD/β-catenin interaction leads to a decrease in the capacity of β-catenin to interact with E-cadherin at the adherens junctions and this decrease plays an important role in tumor invasion since it results in loss of cell-cell adhesion (Yuan et al. 2007). Furthermore, this MUC1-CD/β-catenin complex contributes to β-catenin stabilization by blocking GSK3β-mediated phosphorylation and consequently its degradation in the proteasome (Huang et al. 2005). MUC1-CD/β-catenin is translocated to the nucleus, which has been observed in MUC1-transfected human pancreatic cancer cell lines and rat fibroblasts, and can enhance the activity of β-catenin in association with TCF/LEF transcription factors, promoting cell proliferation and survival through upregulation of *Wnt* target gene transcription (Behrens et al. 1996, Huang et al. 2003, Li et al. 2003, Wen et al. 2003, Baldus et al. 2004). More recently, MUC1-CD/β-catenin complex was associated with a metastatic gene expression signature and an EMT phenotype of tumor cells (Gnemmi et al. 2014). In this work, $CD133^+$ tumor xenografts showed the presence of MUC1-CD/β-catenin interactions in the nuclei of the cells, where they presumably bind to transcription factors and activate transcription of genes involved in cell proliferation and survival such as *cyclin D1* (Huang et al. 2003, Lamb et al. 2003, Udhayakumar et al. 2007). We further showed, for the first time, that pancreatic $CD133^+$ cells exhibit enhanced MUC1 expression and associated signaling partners. CD133 and MUC1 expression are associated with a more aggressive tumor phenotype, in part through enhanced MUC1-CD/β-catenin interactions, and this may contribute to explain the behavior of pancreatic CSCs.

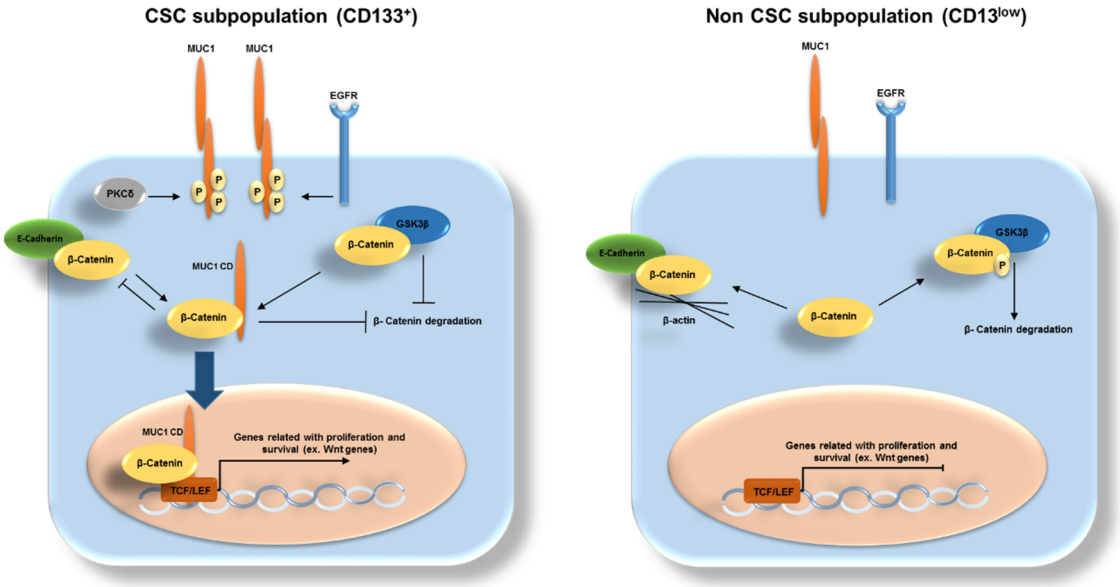

Figure 1. Proposed model for MUC1 signaling partners interactions in the pancreatic CSCs subpopulation. In this model we propose a mechanism for the phenotype of the pancreatic CSC subpopulation through MUC1 signaling pathway. In the CSCs subpopulation ($CD133^{+}$ cells), higher levels of EGFR and PKCδ kinases lead to an increase in MUC1-CD phosphorylation which promotes MUC1-CD/β-catenin interaction. MUC1-CD/β-catenin interaction together with the decrease in GSK3β-mediated phosphorylation stabilizes β-catenin by blocking its degradation in the proteasome. Furthermore, this interaction decreases E-cadherin/β-catenin complex formation at the cell membrane that results in loss of cell-cell adhesion. In the cytoplasm, MUC1-CD/β-catenin complex is able to translocate to the nucleus where it binds to TCF/LEF transcription factors promoting cell proliferation and survival through Wnt/β-catenin signaling pathway activation and therefore contributing to the tumorigenic potential of pancreatic CSCs. The canonical interactions depicted in the $CD133^{low}$ cell subpopulation are responsible for the absence of malignance.

MUC1 structure has been extensively studied since its cloning in 1990. Nevertheless, much remains unknown about the alternative splicing events, and despite more than 70 isoforms have now been described, their specific biological functions and expression profiles remain to be elucidated (Zhang et al. 2013). Alternative splicing events can increase the cell proteome and has been considered more reliable in discriminating tumor from normal tissue than transcription-level differences (Li et al. 2006, Zhang and Gish 2006). MUC1 gene comprises seven exons and six introns and a variety of isoforms can be generated by alternative splicing, intron retention and exon skipping, being exon 2 the most skipped one (Zhang et al. 2013). Therefore, it is crucial to develop knowledge about the specific expression pattern and biological relevance in normal and neoplastic context. Among the differences of expression between different tumors it is important to refer the reported function of MUC1/Y in cell signaling through its phosphorylation, working in a similar way to cytokine receptors (Baruch et al. 1999). It is also important to highlight the described role of MUC1 truncated fragment MUC1* in the induction of EMT, in resistance to standard chemotherapeutic agents

and its potential of being an accurate marker of pluripotency in human embryonic stem cells (Hikita et al. 2008, Fessler et al. 2009, Horn et al. 2009). The lack of a clear understanding about the expression and the biological function of all the described splice variants is mostly due to the absence of specific antibodies.

In this part of the work the objective was to study the relevance of MUC1/S2 splice variant in pancreatic carcinogenesis. This splice variant was identified in human cervical (HeLa) and breast cancer (MCF7) cell lines and is absent in T-cell leukemia Jurkat cells and human activated T cells (Zhang et al. 2013). MUC1/S2 has a retention of 27bp of intron I and excision of 659bp from exon 2 and total excision of exons 3, 4 and 5, lacking the transmembrane domain and cleavage site of the SEA domain. There are no studies about the biological function of this splice variant. A cell line immortalized with the catalytic subunit of telomerase was used, as described by Campbell and colleagues, transformed by sequential retroviral mutant K-Ras (G12D), E6/E7, and SV40 small t antigen transduction cell line, in order to provide some of the oncogenic insults present in the currently accepted pancreatic cancer progression model (Hruban et al. 2000, Campbell et al. 2008). Transduction with MUC1/S2 and MOCK was performed through lentivirus system and stable clones established by limited dilutions. We evaluated the expression pattern of the MUC1/S2 splice variant and its impact in proliferation, migration and invasion of the generated cells. Moreover, we evaluated the subcellular localization of MUC1/S2 and its interaction with p53 protein to evaluate its possible involvement in cell signaling. MUC1/S2 lacks the transmembrane domain making it unlikely to be activated in response to the external environment as it occurs when MUC1 is localized in the membrane (Schroeder et al. 2001). Despite the expression of this isoform being mostly restricted to the cytoplasm it was also possible to observe expression in the nuclei of the cells. MUC1/S2 localization in the nuclei indicates that although not having the cleavage region, MUC1/S2 is capable of translocation to the nuclei which points towards its participation in the modulation of transcriptional events that are involved in malignant progression, namely, invasion and metastasis formation in response to, for example, Wnt/β-catenin or STAT pathways activation (Ren et al. 2002, Gao et al. 2009). In the co-localization assay, this isoform was identified as being present in the mitochondria, golgi apparatus, endoplasmic reticulum and lysosomes. The endoplasmic reticulum, where it was possible to observe the majority of the protein in the studied compartments, has a central role in protein biosynthesis, determining the correct folding of a protein which affects both its function and cellular localization and even if the protein will be secreted. Post-translational modifications, such as *N*-glycosylation, are processes that take place in the ER and can be occurring to MUC1/S2, explaining the observed results. Due to the relevance of the mitochondria in apoptosis, and the relevance of this process in carcinogenesis, it is important to study MUC1/S2 presence in this subcellular compartment deeply, where it was also possible to identify co-localization.

We also evaluated the possible impact of MUC1/S2 in the invasive phenotype of the generated cells. Indeed, MUC1/S2 leads to an increased invasion of pancreatic cancer cells. The involvement of full MUC1 in invasion is documented and one of the described mechanisms shows that once translocated to the nucleus, MUC1 is capable of repressing E-cadherin expression and induce expression of EMT inducers such as Snail and Slug (Roy et al. 2011).

Excitingly, we identified for the first time an interaction between MUC1/S2 isoform and p53 protein, which is known to be involved in MUC1 signaling. p53 is a major tumor suppressor gene involved in DNA repair, apoptosis, cell cycle arrest among others and is mutated in over 50% of human cancers (Lane and Levine 2010). We observed an interaction between MUC1/S2 and p53, mostly in the cytoplasm. This interaction was described, in the nucleus, as contributing to the regulation of p53-responsive genes, namely, activation of *p21* and repression of *Bax* transcription, two proteins involved in cell-cycle progression and apoptosis (Wei et al. 2005). There are no studies showing the relevance of the interaction between MUC1 and p53 in the cytoplasm and further studies are warranted to confirm this interaction and if it plays a role in carcinogenesis.

In the review, we characterized in detail the role of MUC1 in carcinogenesis, highlighting recent findings in cell differentiation and uncovering new evidences of the involvement of MUC1 isoforms in the malignant phenotype. Until now, MUC1 has been found to be a useful biomarker for cancer staging follow-up and there is growing evidence of MUC1 pivotal role on tumorigenesis, remaining to be fulfilled the promise to be used as a target therapy. Hence, efforts in understanding more unexplored fields such as MUC1 involvement in cell differentiation and the putative discriminatory power of different isoforms in disease staging and biological phenotype remain essential.

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Chapter 7

Summary and Conclusions

Pancreatic cancer is one of the most aggressive cancers with 5-year survival rates remaining stable in decades and mortality rates being almost the same as incidence. Early diagnosis and new therapeutic targets to overcome resistance mechanisms remain crucial challenges for both researchers and clinicians.

In this work we aimed to better understand the involvement of the MUC1 glycoprotein in the CSC phenotype and to investigate the biological function of MUC1 splice variants in pancreatic carcinogenesis.

The main conclusions of this thesis are:

We showed, for the first time, that putative pancreatic CSC ($CD133^+$) have increased expression of MUC1 and associated signaling partners. CD133 and MUC1 expression are associated with a more aggressive tumor phenotype, in part through production of enhanced MUC1-CD/β-catenin interactions, and this may explain to some extent the behavior of pancreatic CSCs.

We validated the use of an *in vitro* model based on a transformed pancreatic cell line transduced with a MUC1 splice variant to study its relevance in pancreatic carcinogenesis. Excitingly, it was identified for the first time an interaction between MUC1/S2 isoform and p53 protein. The expression of the studied MUC1/S2 splice variant is mostly restricted to the cytoplasm, however, expression in the nucleus of the cell was also possible to observe. Moreover, its distribution profile included important organelles in cell dynamics such as the endoplasmic reticulum and mitochondria. These findings, together with the observed increased invasive phenotype of the MUC1/S2 cells, suggest that MUC1 splice variants might encompass a plethora of biological functions in the neoplastic pancreatic cell.

The results found in this research work reinforce the importance of MUC1 in the tumorigenic behavior of pancreatic cancer cells, more specifically its role in pancreatic cancer stem cells phenotype. Furthermore, the demonstration that a specific MUC1 splice variant is associated with the acquisition of cancer cell hallmarks, will add these molecules to the scenario of elective targets to address both early diagnosis and therapy of pancreatic tumors.